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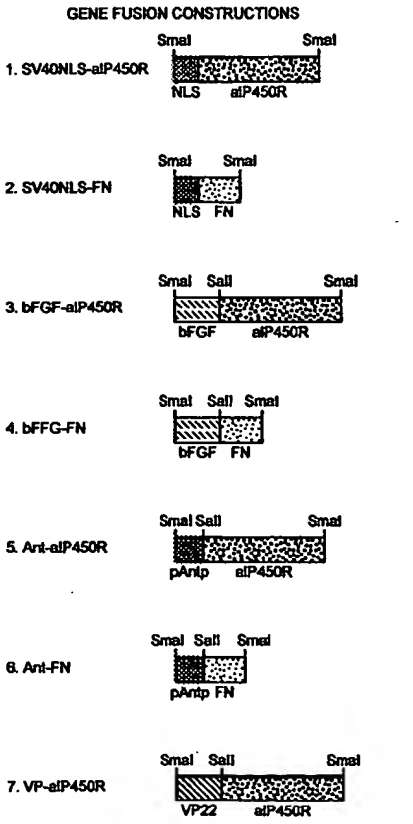
(54) Title: ENHANCED PRODRUG ACTIVATION

(57) Abstract

A prodrug activating agent comprising: a) a localisation domain and b) a prodrug activation domain for activating a prodrug in a target cell.



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Enhanced Prodrug Activation

The present invention relates to an agent for activating prodrugs in target cells. The present invention also relates to methods for activating prodrugs using the novel agents.

Enzyme prodrug therapy (EPT) relates to the use of enzymes to activate prodrugs in therapy. Prodrugs are usually pharmacologically inert or relatively inert compounds, which can be converted *in vivo* to active species having a therapeutic effect (reviewed by Connors 1995 Gene Therapy 2, 702; Springer and Niculescu-Duvaz 1996 Adv Drug Deliv Res 22, 351). One particular use of EPT is in the treatment of tumours.

Initially, prodrugs were developed that exploit the body's innate enzymes to achieve activation, for example Cyclophosphamide (CP) and its isomer, Ifosphamide (IF) are activated by the P450 family of enzymes to produce nitrogen mustards that damage DNA; Mitomycin C (MMC) is activated primarily by NADPH:cytochrome c (P450) reductase to produce a potent alkylating agent (e.g. Bligh *et al* 1990, Cancer Research 50, 7789). The therapeutic index of these first prodrugs depends not upon selective delivery to the target cell but upon the differential susceptibility of the target cells to DNA damage. The damage is only problematic when the cell divides and consequently tumour target cells that are actively dividing, or that will progress to cell division, will be damaged whereas the differentiated cells of the normal organs and tissues to not divide and therefore remain viable. Tumors have not been shown to possess particularly high levels of enzymes that can activate this class of prodrugs (Forrester *et al* 1990 Carcinogenesis 11, 2163). It is thought that prodrugs such as CP, IF and MMC are activated to the therapeutic metabolite predominantly in the liver and the therapeutic metabolite is then distributed to tumour sites via the circulation. Even when tumours possess some capacity to metabolise prodrugs it is thought that down regulation of the activity contributes to drug resistance (e.g. Bligh *et al* 1990 ibid.).

The finding that tumour cells are relatively inefficient at metabolising prodrugs has lead to the concept of delivering normal human enzymes directly to the tumours in order to achieve a high local concentration of the activated compound (e.g. Chen *et al* 1996

Cancer Research 56, 1331). It has recently been shown for example, in experimental systems, that transferring the genes that encode P450 directly into the tum ur cells using viral vectors can enhance the efficacy of CP such that the tumour shows marked growth reduction and regression (Chen *et al* 1996 ibid.). The relevant enzyme activities can be selectively targeted to tumour cells by one of several means. In one approach a tumour selective monoclonal antibody or fragment such as a single chain antibody (scFv) is conjugated to the enzyme either post-synthetically or by producing a recombinant protein. The protein is administered to the patient and localises at the tumour by virtue of specific recognition of the tumour surface by the antibody. The non-toxic prodrug is adminstered and can only be activated at the site of the tumour by the bound conjugate (reviewed by Bagshawe 1987 Br J Cancer 56, 531). This approach is referred to as ADEPT (antibody dependent prodrug activation). Another approach is to deliver the DNA encoding the enzyme to the target cell using either DNA based delivery systems such as plasmids (gene dependent enzyme prodrug therapy GDEPT) (e.g. Vile *et al* 1993 Cancer Res 53, 3860) or using viral vectors (virus dependent enzyme prodrug VDEPT) (e.g. Huber *et al* 1991 PNAS 88, 8039). In these cases tumour specificity may be conferred by incorporating targeting ligands into the delivery vehicle or by using tumour specific promotors (e.g. Harris *et al* 1994 Gene Therapy 1, 170 and refs therein). A recent development describes a combination of the ADEPT and GDEPT approaches whereby a gene fusion is constructed such that the gene encoding the antibody-targeting moiety is fused to the gene encoding the prodrug activating enzyme. The gene fusion is delivered to the target tissue either as DNA or in a gene delivery vehicle such as a viral vector (WO98/55607).

Another class of prodrugs shows a selective toxicity to tumours by virtue of the reductive activation that occurs in the severe hypoxic environment that is a unique physiological feature of tumours. Hypoxia is the condition of abnormally low levels of oxygen and is found in most solid tumours beyond about 1mm in diameter (Coleman 1988 J Nat Canc Inst 80, 310; Vaupel *et al* Cancer Res 49, 6449). The hypoxic environment is conductive to reductive events that can generate reduced derivatives of a variety of chemical groups (Workman and Stratford 1993 Cancer and Metastasis

Reviews, 12, 73-82). The prototypical bioreductive drugs are the antibiotics, Mitomycin C (MMC) and Porfiromycin (POR) but many other compounds are being evaluated including N-oxides such as Tirapazamine (TRZ) (Zeeman *et al* 1986 Inst J Radiot Oncol Biol Phys 12, 1239) and quinones such as the indoloquinone E09 (Bailey *et al* 1992 Int J Radiot Oncol Biol Phys 22, 649) cyclopropamitosenes (EP-A-0868137) and a tertiary amine-N-oxide analogue of Mitoxantrone (AQ4N) that is activated by cytochrome P450 3A4 (Patterson 1993 Cancer Metast Rev 12, 119; Patterson 1994 Biochem Pharm Oncol Res 6, 533). To date the most promising of these compounds is Tirapazamine (TRZ; 3-amino-1, 2,4-benzotriazine-1, 4-dioxide). It is metabolised to a mono-N-oxide (SR4317) and to a lesser extent to the free base SR4330. Neither the two or four electron reduction products are genotoxic and therefore one electron radical of Tirapazamine rather than the stable metabolites is thought to be involved (A. Cahill and I.N.H. White 1990, Carcinogenisis, 11, 1407). It is thought that a nitroxide anion free radical is generated and that this acts directly upon DNA to induce DNA strand breaks (reviewed by J.M. Brown 1993 Br J Cancer 67, 1163). Tirapazamine displays an enhanced cell killing in hypoxic conditions but it also displays some level of toxicity at intermediate oxygen tensions. This is valuable because the degree of hypoxia fluctuates throughout a tumour and absolutely low levels of oxygen may not be maintained for any length of time so endowing Tirapazamine with a greater tumour cell killing potential than for example Mitomycin C which is only activated at very low oxygen tensions (reviewed by J.M. Brown op cit). Tirapazamine does show toxicity at high doses due to effects on the bone marrow resulting in myelo-suppression. Current clinical use is therefore subject to a dose limiting toxicity and in fact the dose required for appreciable therapeutic effect approaches the maximum tolerated dose (Adam *et al* 1992 Int J Radiat Onc Biol Phys 22, 717). Initial work indicated a significant role for cytochrome P450 in the metabolism of Tirapazamine but its role in activation and cytotoxicity are as yet undefined, (Walton *et al* Biochem Pharmacol 44, 251). However, there is increasing evidence that P450 reductase plays a more significant role. Patterson *et al* 1995 Br J Cancer 72, 1144 have shown that in a panel of breast cancer lines there is a range of activities of P450 reductase and the hypoxic toxicity of the Tirapazamine directly correlates with the endogenous P450 reductase levels. Similar correlation has been

made with two bladder carcinoma lines (Xu *et al* 1994 Br J Cancer 242). Recently, formal evidence has been obtained for the importance of P450 reductase in the metabolism of Tirapazamine by showing that the transfer of the full length cDNA for human P450 reductase to, and over expression of the gene for P450 reductase in, tumour cells significantly enhances sensitivity to Tirapazamine (TRZ) (Patterson *et al* 1997 Br J Cancer 76(10), 1338-1347). These data show for the first time that P450 reductase can be used in a gene therapy application.

These studies on the enhancement of prodrug activation by the transfer of normal human enzymes such as P450 and P450 reductase to cells show that normal mammalian enzymes can be exploited in a variety of available strategies. These include the GDEPT, VDEPT and ADEPT approaches described above. Alternatively a haematopoietic cell that has been engineered *ex vivo* to contain the gene can be employed to deliver the enzyme WO98/55607. Direct delivery of prodrug activating enzymes such as P450 and P450 reductase to a tumour allows a reduction in the dose of prodrug that is administered systemically to the patient thus reducing systemic toxicity. All enzyme prodrug therapy approaches rely upon achieving a differential activation of the prodrug in the disease tissue as compared to normal tissue (Chen *et al* 1996; T. Connors op. cit; Patterson *et al* 1997).

Although the exploitation of natural enzymes for EPT strategies is attractive because of, for example, the avoidance of an undesirable immune response, a disadvantage is that for some drugs such as P450 there will still be drug conversion in the liver which could cause some systemic toxicity. A variety of different non-human enzyme prodrug combinations have therefore been explored. These would, by definition, not be present in any human cells and therefore, provided that delivery is specific for the tumour, there will be no toxic side effects derived from prodrug activation in normal tissues such as the liver. For example the thymidine kinase enzyme from herpes simplex virus (HSVTk) can phosphorylate the prodrug Gancyclovir (GCV) to produce a nucleoside analogue which can be subsequently further phosphorylated by cellular kinases to produce a nucleotide (Gancyclovir triphosphate) that functions to block DNA synthesis

(F.L. Moolten 1986 Cancer Res 46, 5276). The HSVTk approach is S-phase specific such that only actively proliferating tumour cells are killed, and will therefore not be suitable in every case. Another example is the use of *E. coli* nitroreductase which reduces compounds related to CB1954 to produce a DNA damaging metabolite (Anlezark *et al* 1992 Biochem Pharmacol 44, 2289). Many other different enzyme prodrug combinations have been devised, for example Carboxypeptidase G_2, penicillin amidase, alkaline phosphatase, β-lactamase and cytosine deaminase can activate prodrugs by hydrolysis (reviewed in the introduction in Knox *et al* 1995 Biochem Pharmacol 49, 1641 and by T. Connors op. cit.; Springer and Niculescu-Duvaz 1996 op. cit.).

A number of factors complicate all of the various enzyme prodrug therapeutic strategies that have been described to date. Different drug/enzyme combinations and different delivery modalities have specific problems. In ADEPT for example, the enzyme is delivered to the exterior of the cell yet in most cases the active drug must cross the cell membrane and this imposes chemical design contraints. The active drug must also diffuse throughout the tumour to have an effect. High level activation at one site resulting from the use of a high affinity antibody, may deplete the prodrug from the bulk of the tumour and simply spill the active compound into the blood circulation thus decreasing efficacy and increasing the potential for systemic toxicity. In addition repetitive prodrug adminstration is required. In general therefore ADEPT strategies are flawed because they have the potential to deliver active compound into the circulation. In addition, if intra-cellular cofactors such as NADPH are required for the enzyme activity, surrogate cofactors may have to be co-administered to the extra-cellular environment, this for example limits the exploitation of bacterial nitroreductases in ADEPT strategies (Knox *et al* 1995 Biochem Pharmacol 49, 1641). VDEPT/GDEPT strategies have an advantage if there is an intracellular cofactor because the enzyme will only activate the drug intracellularly and therefore any fortuitous release of the enzyme into the circulation will not result in systemic activation and toxicity. However, GDEPT and VDEPT strategies cannot deliver the relevant gene to all of the cells in a

tumour which means that many cells in the tumour will not be killed so the intra-cellular restriction of the enzyme becomes a distinct disadvantage. In VDEPT and GDEPT strategies it is therefore desirable that there is some method of extending cell killing beyond the originally engineered cell. This is referred to as the "bystander effect" (Freeman *et al* 1993 Cancer Res 53, 5274). Such effects have been observed for example, with HSV Tk, where many cells are killed in addition to the cell containing the Tk gene. The mechanism is however somewhat controversial as the active metabolite, a highly charged tri-phosphate, cannot traverse cell membranes without the aid of metabolic cooperation via gap junctions (reviewed in T. Connors op. cit. and Hamel *et al* 1996 Cancer Res 56, 2697). The Tk "bystander effect" may in fact be the result of a fortuituous and therefore unpredictable, immunological response rather than a real metabolic effect. Bystander effects have been observed with cyclophosphamide activation by P450 in tumours. In this case it has been proposed that the highly toxic acreolin metabolite mediates the effect but does not cause systemic toxicity because it is relatively shortly-lived (Chen and Waxman 1995 Cancer Res 55, 581). Alternatively the neighbouring cells may take up an intermediate metabolite such as 4-hydroxycyclophosphamide. A bystander effect has also been observed with the aziridin, CB 1954, which can be reduced by DT diaphorase or, with much greater efficiency by bacterial nitroreductases to produce a potent alkylating agent (Knox 1988 Biochem Pharmacol 37, 4661; Bridgewater 1997 Hum Gene Therapy 8, 709) although this has not always been observed (Clark *et al* 1997 Gene Therapy 4, 101-110; Patterson and Stratford, unpublished observations). Any bystander effect will be increased in proportion to the concentration of the active metabolite that is generated. It is therefore desirable to enhance the activity of the prodrug activating enzyme in order to increase the concentration of the active metabolite.

Therefore despite the attraction of enzyme prodrug therapy there are a number of disadvantages to each of the approaches that have been used to date. In order to maximise the potential of enzyme prodrug therapy it is important to use a delivery system and an enzyme/drug combination that shows effective target cell specific cell killing. Preferably also the number of target cells destroyed is increased by creating a

large bystander effect that displays minimal systemic toxicity. The present invention aims to address these needs.

According to one aspect of the present invention there is provided a prodrug activating agent comprising:

a) a localisation domain; and

b) a prodrug activation domain for activating a prodrug in a target cell;

and wherein the localisation domain is not a tumor selective antibody.

The localisation domain and prodrug activation domain preferably comprise amino acid sequences, or are in the form of nucleic acid sequences encoding such domains.

In a preferred embodiment the agent is in the form of a fusion protein. By protein we include peptides and polypeptides.

Alternatively the domains may be co-administered. By co-adminstration we do not necessarily mean simultaneous administration. It is possible for one domain to be administered before or after the other.

"Target cell" simply refers to a cell which the agent whether native or targeted or part of a delivery system is capable of transfecting or transducing.

In another aspect of the present invention there is provided a prodrug activating agent comprising at least one expressable nucleic acid sequence coding for a cytochrome P450 wherein the or each nucleic acid sequence is operably linked to one or more constitutive expression control regulatory element(s), or one or more inducible expression control regulatory element(s).

In another embodiment the agent is in the form or a modified haematopoeitic stem cell (MHSC) which comprises the prodrug activating agent or the prodrug activation domain or has been engineered to express it.

Thus according to another aspect of the present invention there is provided a prodrug activating agent comprising a modifed haematopoeitic stem cell (MHSC) comprising at least one expressable nucleotide sequence coding for a prodrug activating domain wherein the or each nucleotide sequence is operably linked to one or more constitutive expression control regulatory element(s), or one or more inducible expression control regulatory element(s).

In a preferred embodiment the prodrug activating domain according to the present invention further comprises a non-cytotoxic bioreductive moiety.

The present invention also provides nucleic acid vectors comprising the nucleic acid sequence in accordance with the present invention.

The present invention also provides a viral vector comprising the nucleic acid sequence of the present invention.

The present invention also provides a prodrug activating agent, a nucleic acid vector, viral vector, or transduced cell of the present invention for use in medicine.

The present invention also provides a pharmaceutical composition comprising a prodrug activating agent, a nucleic acid vector, viral vector, or transduced cell of the present invention for use in medicine.

In a further aspect the present invention provides a method of treatment of a human or animal patient suffering from a condition such as cancer (in particular solid tumours), cerebal malaria, ischaemic heart disease or rheumatoid arthritis or of a condition characterised by ischaemia, hypoxia or low glucose.

In a further aspect, the present invention provides a method of producing a viral strain comprising introducing a nucleic acid sequence of the present invention into the genome

of a virus. Preferably, the method comprises introducing the nucleic acid sequence into the genome by homologous recombination between said genome and a vector of the present invention.

In a still further aspect the present invention provides a method of producing a MHSC comprising introducing a vector of the present invention into a haematopaeitic strain cell (HSC).

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example with reference to the accompanying drawings in which:

Figure 1A shows P450 reductase sequence;

Figure 1B shows functional domains of P450 reductase;

Figure 2 shows derivatives of P450 reductase;

Figure 3 shows trans-cellular targeting sequences;

Figure 4 shows human cytochrome P450 2B6;

Figure 5 shows IRES;

Figure 6 shows retroviral vectors;

Figure 7 shows gene fusion constructs;

Figure 8 shows a retroviral vector for conferring multiple drug sensitivity; and

Figure 9 shows the product profile obtained on reduction of quinone by $(CH_3)_2COH$.

Figure 10 shows the results of macrophage delivery of P450;

Figure 11 shows P450 PCR fragment;

Figure 12 shows pegHRELacZ;

Figure 13 shows pBHRELacZ;

Figure 14 shows pBHREp450del;

Figure 15 shows pBHREP450;

Figure 16 shows pegHREP450;

Figure 17 shows pONY4;

Figure 18 shows pONY4.1;

Figure 19 shows pONY4HREP450;

Figure 20 shows pONY4.1HRE450;

Figure 21 shows pEGASUS-4;

Figure 22 shows pEGASUS4P450;

Figure 23 shows pONY4.0P450;

Figure 24 shows pONY4.1P450.

Detailed Description of Drawings

Figure 1A shows P450 reductase (P450R) sequence. SEQ ID NO:1 is mRNA sequence (partial, GenBank accession number: S90469); SEQ ID NO: 2 shows the amino acid sequence.

Figure 1B shows function domains of P450 reductase.

Figure 2A shows anchorless P450R. SEQ ID NO: 3 shows the coding DNA sequence. SEQ ID NO: 4 shows the amino acid sequence.

Figure 2B shows FAD and NADPH binding (FN) fragment in which SEQ ID NO: 5 shows the coding DNA sequence and SEQ ID NO: 6 the amino acid sequence.

Figure 3A shows SV40 large T antigen NLS fragment. SEQ ID NO: 10 shows coding DNA sequence (complement 4442-4422) and SEQ ID NO: 11 the amino acid sequence.

Figure 3B shows basic fibroblast growth factor (bFGF). SEQ ID NO: 19 shows the coding DNA sequence for the 18kD isoform and SEQ ID NO: 20 the amino acid sequence.

Figure 3C shows antennapoedia homeobox peptide (pAntp). SEQ ID NO: 53 shows the coding sequence of the homeobox domain and SEQ ID NO: 54 the amino acid sequence.

Figure 3D shows herpes simplex virus Type 1 (HSV-1) tegument protein VP22. SEQ ID NO: 55 shows coding DNA sequence (complement sequence 106391 ... 105486 of GenBank sequence X14112) and SEQ ID NO: 56 the amino acid sequence.

Figure 3E shows Pseudomonas aeruginosa exotoxin A (PEA). SEQ ID NO: 33 shows coding DNA sequence for domain II and SEQ ID NO: 34 the amino acid sequence.

Figure 3F shows single chain Fv fragment (ScFv) for ST4 with translation initiation signal and secretory signal peptide. SEQ ID NO: 25 shows DNA sequence for the translation initial signal and signal peptide of ST4-ScFv (WO98/55607) and SEQ ID NO: 27 the amino acid sequence.

Figure 4 shows human cytochrome P450 2B6. SEQ ID NO: 45 shows the mRNA sequence and SEQ ID NO: 46 the amino acid sequence.

Figure 5 shows IRES (Internal ribosome entry sequence). SEQ ID NO: 52 shows IRES sequence from FMDV (R100) + *SacI* (at the beginning) and *XhoI* (at the end) linkers.

Figure 6A shows PKAHRE: a MLV based single transcription unit vector. Expression of therapeutic genes is controlled by a hypoxia responsive promoter (3xPGK). The gene encoding the prodrug activating enzyme is substituted for the nlsLAcZ gene shown.

Figure 6B shows COI: an MLV based vector with the CMV enhancer replacing the MLV enhancer (shaded box). The gene encoding the pro-drug activating enzyme is inserted either into the *Bam/Sal/Hpa* polylinker or the *Stu/Xho* polylinker. Alternatively different genes can be inserted into each polylinker.

Figure 8 shows a retroviral vector for conferring multiple drug sensitivity. VP22-FN confers enhanced sensitivity to Tirapazamine. P450-FN confers enhanced sensitivity to

Cyclophosphamide. P450-FN plus VP22-FN confers enhanced sensitivity to Mitomycin C, Tirapazamine and Cyclophosphamide.

PRODRUG ACTIVATION DOMAIN

The prodrug activation domain is typically a prodrug activating enzyme or an active fragment of a prodrug activating enzyme; although it may be any domain which activates a prodrug. An increasing number of prodrug activating enzymes are known in the art and any of these may potentially be employed. Suitable prodrug activating enzymes may be natural or engineered and include cytochrome P450, cytochrome P450 reductase, thymidine kinase, nitroreductase, cytosine deaminase, DT-diaphorase, NADPH:cytochrome c P450 reductase and carboxy-peptidase G_2.

A prodrug activating enzyme may be delivered to a tumour site for the treatment of a cancer. In each case, a suitable prodrug is used in the treatment of the patient in combination with the appropriate prodrug activating enzyme. An appropriate prodrug is administered in conjunction with the vector. Examples of prodrugs include: etoposide phosphate (with alkaline phosphatase, Senter *et al* 1988 Proc Natl Acad Sci 85: 4842-4846); 5-fluorocytosine (with cytosine deaminase, Mullen *et al* 1994 Cancer Res 54: 1503-1506); Doxorubicin-N-p-hydroxyphenoxyacetamide (with Penicillin-V-Amidase, Kerr *et al* 1990 Cancer Immunol Immunother 31: 202-206); Para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with β-lactamase); SR4233 (with P450 Reducase); Ganciclovir (with HSV thymidine kinase, Borrelli *et al* 1988 Proc Natl Acad Sci 85: 7572-7576); mustard prodrugs with nitroreductase (Friedlos *et al* 1997 J Med Chem 40: 1270-1275) and Cyclophosphamide (with P450 Chen *et al* 1996 Cancer Res 56: 1331-1340).

Examples of suitable prodrug activation enzymes for use in the invention include a thymidine phosphorylase which activates the 5-fluoro-uracil prodrugs capcetabine and furtulon; thymidine kinase from Herpes Simplex Virus which activates ganciclovir; a cytochrome P450 which activates a prodrug such as cyclophosphamide to a DNA

damaging agent; and cytosine deaminase which activates 5-fluorocytosine. Preferably, an enzyme of human origin is used.

As previously mentioned this is preferably in the form of a prodrug activating enzyme. The prodrug activating enzyme used in treatment of a patient will be administered in combination with the appropriate prodrug.

Preferably the enzyme is a cytochrome P450 or cytochrome P450 reductase.

Cytochrome P450s are a superfamily of membrane-bound, heme-thiolate proteins (Nelson et al 1996 Pharmacogenetics 6: 1-42) that are present in both prokaryotes and eukaryotes. These enzymes are named as cytochromes because they form a complex with carbon monoxide under reductive conditions to yield a maximum absorbance at about 450nm. Within the cell, they are detected either in the endoplasmic reticulum (microsomes) or mitochondria. They are the key monooxygenases responsible for steroid hormones metabolism, drugs inactivation, toxins/xenobiotics detoxification and oxidation of food chemicals, environmental pollutants, carcinogens and other natural or synthetic chemicals. Moreover, some cytochrome P450s have additional isomerases, reductases, dehydrases, nitric oxide synthases or esterases activities (see Rendic & Di Carlo 1997 Drug Metab Rev 29 : 413-580).

Being a terminal oxidase in an electron transport chain, the enzymatic action of cytochrome P450 involves the donation of two electrons from reduced pyridine nucleotide such as NADPH. This electron transfer process requires the interaction of P450 with its redox partners (flavoproteins) NADPH-dependent cytochrome P450 oxidoreductase and cytochrome b_5. Reduced cytochrome P450 binds to oxygen and its substrate. Then this enzyme catalyses the incorporation of one oxygen atom into its substrate and the other atom of oxygen into water. One typical enzymatic reaction is the incorporation of a hydroxyl group by P450 into the substrate e.g. 4-hydroxylation of cyclophosphamide by cytochrome P450 2B6. Furthermore, the expression of cytochrome P450 protein and enzyme activity can be induced or inhibited by certain

xenobiotics, organic substrates or drugs e.g. induction of CYP2B isoforms by barbiturates.

A definition of this enzyme includes the following:

A mono-oxygenase

A heme thiolate

Membrane associated in nature

Enzyme activity requires an electron transport chain

Requires molecular oxygen

The term cytochrome P450 as used herein includes mammalian cytochrome P450 genes such as P450 2B1, P450 2B6, P450 2A6, P450 2C6, P450 2C8, P450 2C9, P450 2C11 and P450 3A4. Each of these genes has been linked to activation of the anticancer drugs cyclophosphamide or ifosphamide.

Generation of P450 mutants for improved P450-based enzyme prodrug therapy

The enzyme activity of cytochrome P450 can be improved by random site-directed mutagenesis or directed evolution. Site-directed mutagenesis requires thorough understanding of the effects of amino acid substitution on the structure and function of cytochrome P450. In contrast, such prior knowledge of P450 is not crucial for directed evolution. Moreover, the frequency of beneficial mutations is generally low relative to deleterious mutations. Multiple cycles of recombination and selection may be required to combine the beneficial mutations for the improved gene. Directed evolution especially DNA shuffling is therefore superior to random mutagenesis because multiple beneficial mutations rather than single beneficial mutation can be generated per round. Nevertheless, both approach requires powerful and efficient selection or screening methods to obtain superior mutants from libraries of millions of different mutants.

Directed evolution can be performed by random oligonucleotide mutagenesis (Horwitz & Loeb 1986 PNAS 83:7405-9), chemical mutagenesis (Sweasy & Loeb 1993 PNAS

90:4626-30), error-prone PCR (Cadwell & Joyce 1994 PCR Methods Appl. 3: S136-40) or DNA shuffling (Stemmer 1994a Nature 370:389-91; Zhao & Arnold 1997 PNAS 94: 7997-8000). All these methods generate libraries of DNA inserts which can be ligated into expression vector for selection or screening in bacterial or mammalian cell settings. However, techniques other than DNA shuffling are limited by the size of DNA insert that they can manipulate. In one typical study, 1-kb fragment has been found to be too large to be generated error-prone PCR whilst DNA shuffling is capable of reassembling DNA fragments up to 2.7kb in size (Stemmer 1994b PNAS 91: 10747-51).

Since the coding sequences of most P450s are bigger than 1-kb, DNA shuffling is the appropriate method for gene improvement of P450. Using Stemmer's method (Stemmer 1994a Nature 370:389-91; Stemmer 1994b PNAS 91:10747-51), related P450 coding sequences are randomly fragmented by DNase I. Fragments of 10-50bp in size are pooled and purified. Overlap extension of these fragments are carried out by repeated thermocycling without primers, in the presence of a DNA polymerase. Full length genes are then obtained by PCR in the presence of specific 5'- and 3'-primers. In addition, point mutations are also introduced in this process by the polymerase. Alternatively, Arnold's staggered extension process (StEP, Zhao et al 1998 Nat Biotechnol 16:258-61) can be employed. Related P450 coding sequences are denatured and primed with defined 5'- and 3'-primers. The primers then undergo a very brief extension catalysed by a DNA polymerase. These extended primers are thermally separated from the templates and are later allowed to anneal back to the templates randomly. In each cycle of denaturation and annealing/extension some of the growing fragments are randomly anneal to different templates (template switching) thus leading to the generation of recombinants. This recombination cycle is repeated until full length genes are produced.

As a smaller scale example, the coding DNA sequences of related human P450 isoforms which are known to be able to metabolise oxazaphosphorines e.g. cyclophosphamide can be shuffled to obtain a better enzyme for this prodrug. This includes **CYP2A6** (Yamano et al 1990 Biochemistry 29: 1322-9 & Genbank accession

number M33316), **CYP2B6** (Yamano et al 1989 Biochemistry 28:7340-8 & Genbank accession number M29874), **CYP2C8** (Okino et al. 1987 J Bi l Chem. 262: 16072-9 & Genbank accession number M17397), **CYP2C9** (Romkes et al 1991 Biochemistry 30: 3247-3255; erratum 1993 Biochemistry 32:1390 & Genbank accession number M61855 or M61857), **CYP2C18** (Goldstein et al 1991 Biochemistry 30, 3247-3255 & Genbank accession number M61856), **CYP2C19** (Romkes et al 1991 Biochemistry 30: 3247-3255; erratum 1993 Biochemistry 32:1390 & Genbank accession number M61854) and **CYP3A4** (Gonzalez et al 1988 DNA 7: 79-86 & Genbank accession number M18907; Beaune et al 1986 Proc. Natl. Acad. Sci. U.S.A. 83: 8064-8068 & Genbank accession number M14096; Spurr 1989 Hum. Genet. 81: 171-4 & Genbank accession number X12387; Bork et al 1989 J. Biol. Chem. 264: 910-919 & Genbank accession number J04449).

For a larger scale approach, two or more subfamilies of mammalian P450 may be shuffled to yield an improved enzyme for cyclophosphamide or other related prodrugs. We include rat **CYP2A1** (Matsunaga 1990 Biochemistry 29: 1329-41 & Genbank accession number M33312), mouse **CYP2A4** (Squires and Negishi 1988 J. Biol. Chem. 263: 4166-71 & Genbank accession number M19319), human **CYP2A6**, rabbit **CYP2A10** (Peng et al 1993 J. Biol. Chem. 268: 17253-60 & Genbank accession number L10236), rat **CYP2B1** (Fujii-Kuriyama et al 1982 Proc. Natl. Acad. Sci. U.S.A. 79: 2793-7 & Genbank accession number J00719), rabbit **CYP2B4** (Gasser et al 1988 Mol. Pharmacol. 33: 22-30 & Genbank accession number M20856), human **CYP2B6**, mouse **CYP2B9** (Noshiro 1988 Biochemistry 27: 6434-6443 & Genbank accession number M21855), dog **CYP2B11** (Graves et al 1990 Arch. Biochem. Biophys. 281: 106-115 & Genbank accession number M92447), rabbit **CYP2C1** (Leighton et al 1984 Biochemistry 23: 204-210 & Genbank accession number K01521), human **CYP2C8**, human **CYP2C9**, rat **CYP2C11** (Yoshioka et al 1987 J. Biol. Chem. 262: 1706-11 & Genbank accession number U33173 or J02657), human **CYP2C18**, human **CYP2C19**, monkey **CYP2C20** (Komori et al 1992 Biochim. Biophys. Acta 1171: 141-6 & Genbank accession number S53046), hamster **CYP2C25** (Sakuma et al 1994 Mol. Pharmacol. 45: 228-36 & Genbank accession number X63022), mouse **CYP2C29**

(Matsunaga et al 1994 Biochim. Biophys. Acta 1184: 299-301 & Genbank accession number D17674), goat **CYP2C31** (Zeilmaker et al 1994 Biochem. Biophys. Res. Commun. 200: 120-5 & Genbank accession number X76502), pig **CYP2C42** (Nissen et al 1998 Anim. Genet. 29: 7-11 & Genbank accession number Z93098), rat **CYP3A1** (Gonzalez et al 1985 J. Biol. Chem. 260: 7435-7441 & Genbank accession number M10161), human **CYP3A3** (Molowa et al 1986 Proc. Natl. Acad. Sci. U.S.A. 83: 5311-5 & Genbank accession number D00003), human **CYP3A4**, human **CYP3A5**, human **CYP3A7** (Komori et al J. 1989 Biochem. 105: 161-3 & Genbank accession number D00408), monkey **CYP3A8** (Komori et al 1992 Biochim. Biophys. Acta 1171: 141-6 & Genbank accession number S53047) and dog **CYP3A12** (Ciaccio et al 1991 Biochim. Biophys. Acta 1088: 319-322 & Genbank accession number X54915). However, the capabilities of some of the above listed isoforms to metabolise oxazaphosphorines are still unknown.

LOCALISATION DOMAIN

Intracellular localisation domain

In a first embodiment the localisation domain is an intracellular localisation domain.

The intracellular localisation domain serves to associate the prodrug activation domain with a sub-cellular location and so increases the effectiveness of the prodrug activation domain. This in turn reduces the dose of prodrug that is required and so increases the therapeutic index.

In this preferred embodiment, the target site in the target cell is a sub-cellular site of action of the activated prodrug. The fusion protein therefore delivers the prodrug activation domain to the relevant location in the target cell. This improves the effectiveness of the activated prodrug.

The intracellular localisation domain is typically a peptide or polypeptide which is capable of preferentially or specifically binding at a sub-cellular location, such as an

organelle. For example, the intracellular localisation domain may have a specific binding partner in an intracellular membrane such as the nuclear membrane r the mitochondrial membrane. Alternatively or additionally, the intracellular localisation domain may be specifically taken up by an organelle within the target cell, such as the nucleus or the mitochondria. Thus preferably the intracellular localisation domain is a nuclear localisation domain. Nuclear localisation domains suitable for use in accordance with the invention include nuclear localisation signals from the SV40 large T antigen, nucleoplasmin and c-myc.

It is believed that the effectiveness of the active prodrug will be proportional to the concentration of the active metabolite at the sub-cellular site of action. Many of the active compounds mediate their toxic effect via direct effects on the DNA of the tumour cell. Free radicals cause DNA strand breakage as is the case with activated Tirapazamine (TPZ) (Cahill and White op. cit.) whereas alkylating agents such as CP, Mitomycin C and CB1954 cross link DNA leading to shear upon replication (Bligh *et al* 1990, Cancer Res. 50, 7789; Knox *et al* 1988, Biochem. Pharmacol. 37, 4661). Given the short half life of some of these compounds there has been speculation that some of the activating enzymes might be found in the nucleus (J.M. Brown op. cit.) and indeed both cytochrome P450 and P450 reductase activity can be detected in nuclear fractions (Cahill and White op. cit.; Friedman *et al* 1989 Biochem. Pharmacol. 38, 3075). We have shown that when the full length P450 reductase cDNA is transferred to target cells the P450 reductase protein localises at the endoplasmic reticulum (ER) within the cytoplasm [A.V. Patterson 1997 op. cit. Fig. 1]. the protein has an ER membrane anchor sequence and is normally found at this site (G.C.M. Smith *et al* 1994 Proc. Natl. Acad Sci 91, 8710). The active part of the enzyme projects into the cytosol and therefore activated metabolites will be generated at the ER and should then be free to passively diffuse into the nucleus. Deletion of the ER anchor sequence does not interfere with the folding of the protein or its ability to bind co-factors and donate electrons (Smith *et al* op. cit.). P450 reductase is naturally a membrane associated component of the ER, however we considered that it might be possible to retarget the enzyme to the nucleus and further, to the chromatin in order to achieve an increase in

the local activation of the prodrug at the site of the target for toxicity. Thus, in a preferred embodiment the intracellular localisation domain is a nuclear association domain. A first step to enhance delivery of the metabolite to the DNA is to target the P450 reductase to the nucleus. A diverse variety of peptide signals have been identified that mediate the import of proteins into the nucleus (reviewed by E.A. Nigg 1997, Nature 386, 779) including but not restricted to nuclear localisation signals identified in the following proteins :-

SV40 large T antigen	PKKKRKV
Nucleoplasmin	KRPAATKKAGQAKKKK
c-myc	PAAKRVKLD

[signals are written as the conventional single letter amino acid code].

The ER anchor sequence of P450 reductase (defined as residues 1 to 60, T.D.Porter *et al* 1990, Biochem 29, 9814) is removed to produce an anchorless P450 reductase (alP450 reductase). If the anchor is replaced by a nuclear localisation signal (NLS) the P450 reductase is no longer localised to the ER but is found predominantly in the nucleus. Furthermore, the increased local concentration of P450 reductase in the nucleus increases the sensitivity of the cells to killing by Tirapazamine (TPZ). The derivative is referred to as NLS-alP450 reductase.

It has been shown that the P450 reductase gene is organised into functional domains that correspond to the exon structure of the gene. In particular domains that bind FMN, FAD and NADPH have been identified (T.D. Porter *et al* 1990 Biochem. 29, 9814). These domains are structurally and functionally discrete such that they can be expressed as protein fragments and still bind their respective cofactors. It has been shown for example that a peptide spanning residues 242 to 677 will bind FAD and NADPH and furthermore this domain (the FN fragment) can transfer electrons to a range of one-electron acceptors (Smith *et al* op. cit.). To increase the efficiency of nuclear targeting the FN fragment is used that contains only the active region involved in electron transfer to exogenous electron acceptors. The nuclear targeting signal is fused to residues 242 to

677 of P450 reductase. The resulting smaller protein has easier access to the nucleus. An additional benefit is that electrons are not 'lost' by transfer to FMN and to P450 and are therefore available for transfer to exogenous electron acceptors. A further benefit is that the smaller size makes room for additional genes in certain GDVs e.g. retroviral vectors where capacity is restricted. The derivative is referred to as NLS-FN.

P450 reductase is a charged molecule and contains lysine and arginine rich regions (E.A.Shephard *et al* 1992, Arch. Biochem. BioPhys., 294, 168). This positive charge may favour retention in the nucleus and may even locate the protein at the DNA. However in order to optimise nuclear retention and chromatin localisation even further other fusions can be envisaged. These could for example be fusions with transcription factors, high mobility group proteins, nuclear envelope proteins or any protein with a desirable nuclear localisation.

Transcellular localisation domain

In a second embodiment the localisation domain is a transcellular localisation domain.

In this aspect the localisation domain delivers the prodrug activation domain to neighbouring cells so increasing the number of cells that can be treated. An amplification of the effects of a prodrug beyond the cell in which it is activated is called the "bystander" effect. This has previously been achieved by the diffusion of the active metabolite to neighbouring cells. This approach has two main disadvantages in that it will concomitantly deliver the metabolite systemically, so risking systemic toxicity and there will be a dilution of the metabolite during cell to cell transfer. The present invention is particularly useful for those prodrugs where the active metabolite is poorly diffusible across membranes e.g. the nucleotide analogues generated by thymidine kinase and cytosine deaminase, the nitrogen mustards generated by cytochrome P450 metabolism, the Tirapazamine radical generated by P450 reductase. This is because it provides for the activating enzyme to cross membranes rather than the metabolite. Useful transcellular activation domains include the third helix domain of the Drosophila

antennapoedia homeobox peptide, the Herpes simplex virus VP22 protein, basic fibroblast growth factor (bFGF/FGF2), secreted single chain antibodies (s.scFv) and the transmembrane transport signals from toxins such as the Pseudomonas aeruginosa exotoxin (PEA). Also useful are membrane translocating sequences such as Kaposi fibroblast growth factor and transcription factor kB.

The active metabolite of Tirapazamine (TPZ) is sufficiently labile to limit any bystander effect and this diminishes the effectiveness of this enzyme in VDEPT or GDEPT strategies where a significant number of cells might not receive the gene. In order to increase the bystander effect of drugs such as Tirapazamine (TPZ) we have devised novel approaches for distributing the enzyme throughout the target tissue rather than distributing the metabolite as has been done previously. To achieve this the ER membrane anchor is replaced with a trans-cellular targeting signal (TTS) that ensures export from the cell and import into neighbouring cells. In one embodiment the TTS also confers target cell specificity.

Export/import or trans-cellular targeting signals (TTS) can be obtained from a diverse array of biological systems and are not necessarily mechanistically related in the manner in which they mediate export-import.

Some examples of suitable TTSs are as follows:-

- Basic fibroblast growth factor (bFGF or FGF2). In many cells this is secreted despite lacking a classical secretion signal sequence. The bFGF receptor is up-regulated in many tumour types and interaction with bFGF results in rapid internalisation of the ligand and any macromolecule that is linked to it [Baldin *et al* 1990 EMBO J. 9, 1511; Sosnowski *et al* 1996, J. Biol. Chem. 271, 33647 and refs cited therein]. Fusion of bFGF to P450 reductase or the FAD/NADPH fragment produces a protein that is exported and that is taken up by any cell displaying the FGF receptor. The complex is transported to the nucleus. Additional signals can be added such as a classical secretion

signal and NLS to ensure efficient export form any producer cell and efficient nuclear import in any target cell. The derivatives are bFGF-P450R and bFGF-FN.

- pAntp. This is a 60 amino acid polypeptide corresponding to the homeobox domain contained in the third exon of the Drosophila antennapoedia protein. It has the property of penetrating cells and transferring to the nucleus (Joliot *et al* 1991 Proc. Nat. Acad. Sci., 88, 1864). Smaller peptides derived from pAntp also have import activity and fusion proteins can be internalised (Derossi *et al* 1994. J. Biol. Chem. 269, 1044). It is not clear how well this peptide is exported from cells and therefore a classical secretion signal is included in the fusions. The anchorless P450 reductase is fused to a secretion signal sequence to mediate export to the homeobox domain of the Drosophila antennapoedia protein to mediate import into the nucleus of neighbouring cells. The derivatives are Ant-P450 reductase and Ant-FN.

The VP22 protein from herpes simplex virus. This is a 301aa virion structural protein that is naturally exported from infected cells, imported by neighbouring cells, transported to the nucleus and docked onto chromatin. Proteins that are fused to VP22 are similarly exported/imported and located. [Elliott and O'Hare 1997; WO97/05265]. The anchorless P450 reductase is fused to VP22 and the fusion protein is distributed to the nuclei of neighbouring cells. The derivatives are VP-P450 reductase and VP-FN. Preferably the VP22 and P450 reductase domains are separated by a flexible linker (Somia *et al* see later).

-Truncated Pseudomonas aeruginosa exotoxin A (PEA) encoding the translocation domain (II) (I. Pastan and D. Fitzgerald 1991 Science, 254, 1173; J. Hwang *et al* 1987, Cell, 48, 129). The translocation domain is fused to a targeting ligand such as a single chain antibody preferably one which recognises a tumour cell specific antigen (e.g. S.I. Chen *et al* 1997 Nature, 385, 78 and refs therein) and to the NLS-alP450 reductase. The fusion protein is secreted, docked onto the tumour cell surface, translocated and imported into the cell. The derivatives are PEA-5T4-P450 reductase and 5T4-PEA-FN. By way of example the targeting ligand is a single chain antibody directed to the

oncofetal antigen 5T4, but any scFv could be used for example the scFv against HER-2 (J.K. Batra *et al* 1992, Proc Natl Acad Sci 89, 5867). The invention is not necessarily restricted to the use of single chain antibodies, it may be preferable in some cases to use a two chain antibody Fab domain.

- Synthetic or natural membrane translocating sequences (MTS). Another suitable trans-cellular targeting domain is the hydrophobic region of a signal peptide, usually referred to as a membrane translocating sequence (MTS). These are found in many natural genes for example transcription factor kB, Kaposi fibroblast growth factor or they can be synthetic peptides selected for an MTS function. These MTSs can be used as carriers to deliver short peptides into living cells (e.g. Lin *et al* 1995. Inhibition of nuclear translocation of transcription factor NG-kB by a synthetic peptide containing a cell membrane permeable motif and nuclear localisation sequence. J. Biol. Chem 270, 14255). Such peptides can be placed at the N- or C-terminus of a peptide and have been shown to be capable of translocating full length proteins (e.g. Rojas *et al* 1998, Genetic engineering of fusion proteins with cell membrane permeability, Nature Biotechnology, 16, 370). For example the Kaposi fibroblast growth factor MTS was fused to a bacterial enzyme encoding glutathione-S-transferase. The MTS-enzyme protein was expressed in E.coli and the purified protein was shown to be readily taken up by mouse cells.

We now show how to use an MTS in a gene therapy application. The MTS is fused to all or the active part of prodrug activating enzyme such as P450 reductase or nitroreductase preferably to a derivative of such an enzyme for example a 5T4scFv fusion protein that can be secreted from the cell of production. The resulting fusion protein is taken up by surrounding cells so increasing the number of cells capable of activating a prodrug. The secretion signal is not necessarily associated with a targeting ligand such as an scFv and any suitable secretion signal sequence can be used. The addition of a secretion signal allows release of the fusion protein from the production cell in situations where the cell does not, naturally or as a consequence of cell death, liberate proteins.

Biochemical association domain

In a third embodiment the localisation domain is a biochemical association domain. By biochemical association domain we mean a second prodrug activation domain such that products of one prodrug activation domain are delivered to another prodrug activation domain. In a preferred embodiment the additional domain may be another enzyme such that the products of the first enzyme are directly delivered to a second enzyme. This achieves biochemical association that may or may not be combined with nuclear and trans-cellular targeting. An example of biochemical association is where cytochrome P450 and NADPH cytochrome reductase are involved in the prodrug metabolism. P450 reductase donates electrons to cytochrome P450 and the biochemical reaction is enhanced by proximity. Making a fusion between the two proteins can increase proximity so enhancing the efficiency of electron transfer to adjacent protein domains. This is useful, for example, for the activation of Cyclophosphamide which can be enhanced by the provision of excess cytochrome P450 (e.g. Chen *et al* 1996, Cancer res. 56, 1331; Patent Publication No. WO 96/04789) but which will be rate limited by P450 reductase which is known to be sub-optimal for cytochrome P450 activity even when the enzymes are in equi-molar ratios (e.g G. Truan *et al* 1993 Gene, 125, 49). Enhancement of the activity of cytochrome P450 by biochemical association with P450 reductase has been described in bacterial systems (A. Parikh *et al* 1997, Nature Biotechnology, 15, 784) but has not been proposed for gene therapy.

Certain prodrugs might be metabolised to different products depending upon the prodrug activation domains involved in the transformation or combinations of prodrug activation domains might potentiate the metabolism of a prodrug. For example, there is some evidence that both P450 and P450 reductase contribute to the hypoxic toxicity of Tirapazamine (Walton *et al* 1992 op. cit., J.M. Brown ip. cit.). Similarly, the ability of P450 to hydroxylate cyclophosphamide is dependent upon the transfer of electrons from P450 reductase suggesting that an elevated activity of both enzymes could enhance prodrug metabolism. We show that a combination of prodrug activation domains can maximise the toxic potential of one or more prodrugs. The configuration of the

combination will be dictated by the particular prodrug combination. A number of configurati ns are outlined below by way of example:-

The coding region for P450 is fused to the coding region for P450 reductase or to one of the derivatives of P450R as outlined above. Fusions between a cytochrome P450 and P450 reductase have been described previously but not with the specific coding sequences described herein and not in the context of a gene therapy application (Blake *et al* 1996 FEBS Lett 397:210; cytochrome P450 3A4 fused to P450 reductase, M.S. Shet *et al* 1993 Proc. Natl. Acad. Sci. 90, 11748; rat cytochrome P4501A1 fused to yeast P450 reductase, T. Sakaka *et al* 1994 Cytochrome P450 8th Int. conference Ed. M.C. Lechner pp 429-432; Chun *et al* 1996 Arch Biochem Biophys 330:48). The proximity of the proteins in the fusion enhances electron transfer to P450 so increasing the metabolism of prodrugs such as Cyclophosphamide.

The biochemical association domain may also be in the form of a macrophage. In this embodiment the macrophage will express the prodrug activating enzyme.

As indicated above it is preferred if P450 is in proximity to P450 reductase. We have now found that P450 reductase is present in human macrophages. We have now found that P450 is not expressed in macrophages, but we have also found that macrophages can be engineered to express P450. We have further shown that such engineered macrophages can be used to activate prodrugs, such as cyclophosphamide. Other examples of prodrugs are given elsewhere in this description.

Preferably the macrophages are engineered to express the gene encoding the prodrug activating enzyme constitutively or alternatively regulatively. Example of suitable promoters are the cytomegalovirus CMV promoter and the hypoxia response element (HRE); although other promoters may be used and will be known to those skilled in the art.

The prodrug activating agent which may be expressed by the macrophage is not limited to P450. Other prodrug activating agents may be used such as cytosine deaminase. Such agents will be known to those skilled in the art.

In a particularly preferred embodiment of the present invention there is provided a macrophage capable of expressing P450. Preferably the P450 is CYP2B6. Preferably the P450 is under the control of a CMV promoter. Such a prodrug activating agent may be used in therapy in combination with the prodrug cyclophosphamide.

Macrophages may also be used a method of delivery as will be described later.

Chemical association domain

In a fourth embodiment the localisation domain is a chemical association domain. By chemical association domain we mean a domain which alters the intracellular chemical environment to optimse conditions for the activity of the prodrug activation domain. For example the bioreductive activation of certain prodrugs is dependent upon the hypoxic environment. There are drugs such as Tirapazamine where the activation is reversed by molecular oxygen (reviewed by J.M. Brown Br. J. Cancer 1993, 67, 1163). Tirapazamine is activated by NADPH dependent reductases such as P450 reductase. In the invention the additional protein is used to deplete the cell of molecular oxygen such that conditions for bioreduction of Tirapazamine are favoured. One example is the provision of cytochrome P450 enzymes with P450R. The cytochrome P450 is a mono-oxygenase and will reduce molecular oxygen so increasing the hypoxic environment for P450 reductase to activate Tirapazamine. Enhancement of the activity of cytochrome P450 by P450 reductase has been proposed in microbial systems but the enhancement of P450 reductase activity by cytochrome P450 has not been proposed previously in any context.

The chemical association domain may also be used in concert with an intracellular, transcellular or biochemical association domain.

We propose that because P450 utilises molecular oxygen it can be used to further potentiate the activation of drugs such as Tirapazamine (TPZ) and MMC by helping to maintain the hypoxic environment. It has been shown that co-adminstration of Cyclophosphamide with Tirapazamine in mice gives enhanced tumour cell killing (S.A. Holden *et al.* 1992,J. Nat. Canc. Inst 84, 187). Whilst not wishing to be bound by any theory it may be that this is an *in vivo* discovery of the potentiation concept described above. We now show that the co-administration of cytochrome P450 and P450 reductase results in an enhancement of cell sensitivity to Tirapazamine. A further advantage of the combination of cytochrome P450 and P450 reductase is that it has utility for combination drug therapy with Cyclophosphamide and Tirapazamine. Other combinations of prodrug activating enzymes and derivatives are as follows: for example the fusion protein 450FN can be co-expressed with P450 reductase or derivatives to potentiate CP activation and Tirapazamine activation. Other drug combinations include Mitomycin C and Tirapazamine.

Mitochondrial Association

Mitochondrial DNA (mt-DNA) is a highly susceptible target for DNA adduct formation. The mt-DNA genome is saturated by functional and essential coding sequences (94.4%) with very little redundancy, mt-DNA lesions are potentially more cytotoxic than nuclear DNA lesions since the probability of generating damage in a critical coding region is far greater. Furthermore, mt-DNA has no histones to protect the DNA from attack, so it is highly susceptible to alkylation by an active metabolite or a radical-generating strand-breaking agent. Mitochondria have been shown to be an important target for agents such as MMC (Pritsos *et al* 1997 Oncol Res 6-7, 333).

Evidence also points to a central role for mitochondria in apoptotic response (Henkart and Grinstein 1996 J Exp Med 183, 1293; Papa and Skulachev 1996; Decaudin *et al* 1998 Int J Oncol 12, 141). The nuclear features of apoptosis are preceded by alterations in mitochondrial function and structure (Petit *et al* 1997 Mol Cell Biochem 174, 185).

This appears to involve the release of a protease-like apoptosis-inducing protein that is normally sequestered in the intermembrane space. It is released in response to disruption of mitochondrial membrane due to swelling of mitochondria caused by opening of so-called permeability transition pores in their inner membrane. Crucially, increases in the level reactive oxygen species (ROS) is known to induce the mitochondrial pores (Skulachev 1996 FEBS Lett 397, 7; Susin *et al* 1997).

It is envisaged that targeting of a prodrug activating enzyme to the mitochondrion would increase the toxicity of prodrugs to the cell and would stimulate the apoptotic response pathway.

A first step to enhance the delivery of a prodrug metabolite to mitochondrial DNA is to import the prodrug activating enzyme into the mitochondrion. Mitochondrial import pathways are well described. Mitochondrial proteins are synthesised as preprotein precursors containing an amino-terminal presequence. Few proteins are localised to mitochondria without such amino-terminal extensions. These presequences are necessary and in some cases sufficient for import (Verner and Schatz 1988 Science 241, 1307; Fenton 1995 Amer J Human Genetics 57, 235). Although presequences lack primary sequence homology, they are enriched for both basic and hydroxylated amino acids, lacking both acidic amino acids and long hydrophobic stretches, and may be able to form amphiphilic α-helical and β sheet conformations. In the present invention the DNA sequence encoding a mitochondrial import signal is fused to the coding sequence for the prodrug activating enzyme. Suitable import sequences are described in Verner and Schatz 1988 and Fenton 1995. The fusion protein is expressed in the target cell and the fusion protein is imported into the mitochondria.

BIOREDUCTIVE MOIETIES

The present invention may also employ bioreductive drug conjugates e.g. for use in targeting of therapeutic agents to localised regions of hypoxic and/or ischemic tissue within the body.

Reduced oxygen tension (hypoxia) has been demonstrated in a variety of tumor types. In fact, it has long been suspected that oxygen deficiency in tumors may be a limiting factor in the control of tumors by radiotherapy. Relatively recently, the presence of hypoxia in tumors has been exploited in their treatment.

Bioreductive drugs require metabolic reduction to generate cytotoxic metabolites. This process is facilitated by the presence of appropriate reductases and the lower oxygen conditions present in some cancerous (hypoxic) as compared to normal (normoxic) tissue. As a result, a number of bioreductive drugs capable of producing cytotoxic metabolites under hypoxic conditions have been proposed for use in combination with radiotherapy treatment of tumors.

A number of bioreductive compounds are known to act as potent alkylating agents after undergoing reduction *in vivo*. Examples of known bioreductive alkylating agents include compounds such as activated enamines, vinylogous quinone methides, simple quinone methides and α-methylene lactones or lactams. Bioactivation of such compounds produces species which are electron deficient and which are capable of covalent binding to a nucleophilic centre on a biomolecule, such as DNA.

Most bioreductive drugs that have been developed for use in the treatment of tumors exhibit an optimum "trapping" potential when hypoxia is profound (pO_2 < 12 mm Hg) and this is believed to form the basis for their selectivity for cancerous as opposed to normal tissues.

Bioreductive drugs have also been proposed for use in several meth ds for the detection of hypoxic cells in tumors. In this way, radiotherapy treatment may be optimised for individual patients on the basis of the oxygen status of their tumors.

US-A-5086068 describes the use of nitroaromatic compounds in the detection of hypoxic cells in normal and tumor tissue. An immunogenic conjugate comprising a nitroaromatic compound and an immune response inducing carrier is used *in vitro* to raise antibodies specific to the nitroaromatic compound. These antibodies are in turn used to detect the presence of hypoxic tissue following *in vivo* adminstration of the nitroaromatic compound.

A number of methods have also been described for detecting the presence of hypoxic cells in tumors using a labelled 2-nitroimidazole in which labelled fragments of the nitroimidazole compound bind to cellular macromolecules. More recently, the use of an immunologically detectable hapten such as theophylline covalently bound to a 2-nitroimidazole has been suggested as a method of identifying hypoxic cells (see Brit. J. Cancer 63: 119-125, 1991 & 72: 1462-1468, 1995, and Anti-Cancer Drug Design 10: 227-241, 1995). Bioreduction of the nitroimidazole leads to binding of bioreductive metabolites, and hence the theophylline side-chain, to intracellular molecules. Immunochemical techniques are then used to stain and thus locate those cells containing the bound theophylline.

Other agents comprising a bioreductive moiety, e.g. 2-nitroimidazole, for the diagnosis or treatment of hypoxic cells are described in US-A-5387692.

A number of bioreductive agents have been described for use in the delivery of cytotoxic drugs to hypoxic tumor tissue in which bioreductive activation at the tumor site results in selective delivery of the drug. However, following drug delivery the bioreductive compound remaining in the tissues is itself a potential alkylating agent and thus cytotoxic, thereby rendering such a system entirely unsuitable for use as a non-cytotoxic drug delivery vehicle in diseases other than cancer. Hypoxia-selective

bioreductive drug delivery agents proposed for use in anti-tumor therapy are described, for example, in Dissabs. 87: 31004, 1987 and in J. Med. Chem. 34: 2933-2935, 1991.

Delivery systems which utilise bioreduction to deliver a non-cytotoxic drug species have also been proposed. For example, a delivery system based on quinone propionic acid has been described (see Pharmaceutical Research 8(3): 323-330, 1991) in which the benzoquinone acts as the trigger and the propionic acid moiety allows for linkage either to an amine moiety (e.g. an enzyme inhibitor) or to an alcohol (e.g. a steroid). Two electron activation of the benzoquinone trigger facilitates intramolecular cyclisation generating a stable lactone, a process which results in elimination of the drug species. However, the lactone produced is itself a potential alkylating agent. This system is thus unsuitable for use as a non-cytotoxic drug delivery system. Furthermore, in aqueous solution in the absence of a reducing agent the lactone produced following drug delivery is very unstable and undergoes degradation. The instability of this prodrug system in aqueous solution thus precludes its use for drug delivery *in vivo*.

We now propose an improved method for the specific targeting of a drug to areas of hypoxic and/or ischemic tissue, e.g. cells, tissues and/or organs, within the body in which the desired drug species is linked to a non-cytotoxic bioreductive compound or carrier. In this method, any direct interaction of the carrier with DNA or other biomolecules is minimised, thus avoiding potential mutagenic side effects.

In particular, we now propose a method capable of targeting drugs to sites of inflammation within the body associated with hypoxia and/or ischemia, e.g. to the synovium in the treatment of rheumatoid arthritis. This method not only has the effect of reducing the risk of systemic side effects of the drug, but also enhances the therapeutic effect of the drug.

Thus, viewed from one aspect the invention now provides a bioreductive conjugate comprising a non-cytotoxic bioreductive moiety with linked thereto at least one therapeutic agent.

The bioreductive conjugates useful in the present invention may have the formula (I):

$$A(B)_n \qquad (I)$$

where A is a non-cytotoxic bioreductive moiety, each B is independently the residue of a therapeutic agent, and n is an integer, preferably from 1 to 3, particularly 1.

A and B are stably conjugated in an oxygenated environment and are such that A is non-cytotoxic and B when conjugated to A is non-cytotoxic. On reductive activation of A, A and B detach and A is itself either a stable, non-cytotoxic species or, more preferably, A reacts with itself to form a stable, non-cytotoxic species.

Preferred compounds for use in accordance with the invention are those which have the ability to penetrate poorly perfused tissues and which only release the active drug in a hypoxic and/or ischemic environment.

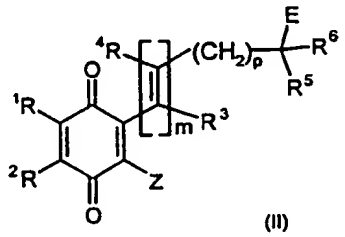
A large number of bioreductive agents of diverse structure are known. These include quinones, aromatic nitro compounds and N-oxides. As mentioned above, those intended for use in accordance with the invention should be substantially non-cytotoxic following bioreductive activation. This may be achieved in a number of ways.

Following bioreduction of the conjugate and delivery of the drug species to the target site, the final form of the bioreductive carrier may itself comprise a stable, non-cytotoxic species, for example a compound having no potential alkylating centre. However, in a preferred embodiment of the invention cytotoxicity of the bioreductive moiety may be reduced by providing a nucleophilic centre within the bioreductive compound itself. Following release of the drug an alkylating centre is formed. However, the proximity of the nucleophilic centre ensures that intramolecular alkylation occurs in preference to alkylation of any biomolecules such as DNA. In this way, substantially no cytotoxic species are formed. Such systems may be referred to as "self-alkylating".

Examples of electron rich groups capable of acting as a nucleophilic moiety in the bioreductive compound include oxygen, sulphur and nitrogen atoms. Thus, for example, inclusion of a suitably positioned amino, thio or hydroxyl group within the bioreductive compound will favour intramolecular alkylation resulting in a non-cytotoxic product on release of the drug at the site of hypoxia/ischemia. Suitable nucleophilic moieties which may be present in the bioreductive moiety include -OH, -SH, $-NH_2$ and -NHR in which R is C_{1-6} alkyl, e.g. C_{1-3} alkyl. Other suitable nucleophilic moieties will be known to those skilled in the art.

Alternatively, the bioreductive compound for use in the invention may be rendered non-cytotoxic following drug delivery by means of the introduction of steric hindrance capable of presenting a physical blockage to attack upon the bioreductive by any nucleophile. Thus, the presence of a bulky group either at or in close proximity to any potential alkylating centre generated in the bioreductive moiety following drug delivery serves to abolish alkylating reactivity thus preventing alkylation of any biomolecules. Examples of groups which may be used in this way include linear or, more preferably, branched, C_{4-20} alkyl or alkenyl groups, e.g. tert. butyl. Other groups capable of providing steric hindrance will be known to those skilled in the art.

Particularly preferred bioreductive conjugates in accordance with the invention include compounds of formula II:



(II)

(wherein

R^1 and R^2 independently represent hydrogen or halogen atoms, or a group R, OR, SR, NHR, NR_2, CO_2R or CONHR;

or, alternatively, R^1 and R^2 together with the intervening ring carbon atoms form a 5-7 membered, preferably 5- or 6-membered, carbocyclic or heterocyclic ring itself

optionally substituted by one or more halogen atoms, or by one or more groups selected from R, OR, SR, NHR, NR_2, CO_2R and CONHR;

Z represents an alkyl, alkenyl, aryl or aralkyl group optionally carrying at least one OH, SH, NH_2 or NHR^7 group in which R^7 is an alkyl group;

R^3, R^4, R^5 and R^6 independently represent hydrogen atoms or an alkyl or alkenyl group;

each group R independently represents a hydrogen atom, an alkyl or alkenyl group;

E represents the residue of a therapeutic agent to be delivered, optionally attached via a linking group L;

m = 0, 1, 2 or 3, preferably 1;

p = 0 or 2, preferably 0;

with the proviso that when m = 1 then p = 0)

or a salt thereof.

Preferred compounds of formula II include those wherein Z represents a group of the formula $(CH_2)_nXH$ in which n = 0, 1, 2 or 3, preferably 0; and X represents an oxygen or sulphur atom or, preferably, X represents a group of formula NY wherein Y represents a hydrogen atom or an alkyl group. Such compounds may act as "self-alkylating" systems.

Particularly preferred compounds of formula II are those wherein Z represents a group of the formula $(CH_2)_nXH$ in which X represents an amino group;

R^1 and R^2 each represent alkoxy groups or, together with the intervening ring carbon atoms, R^1 and R^2 form a benzene ring;

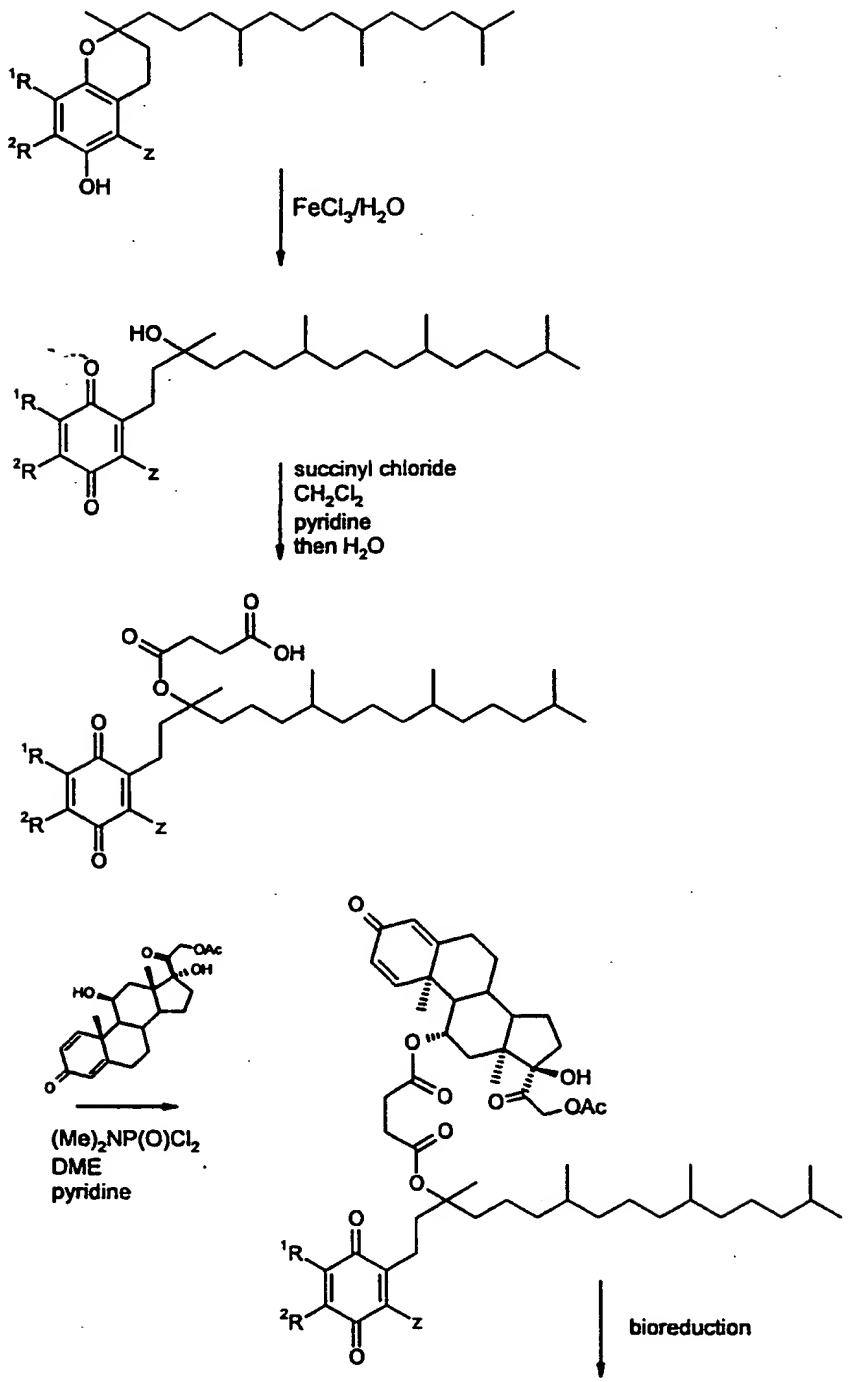
R^3, R^4, R^5 and R^6 each represent hydrogen atoms; and

n = 0, m = 1 and p = 0.

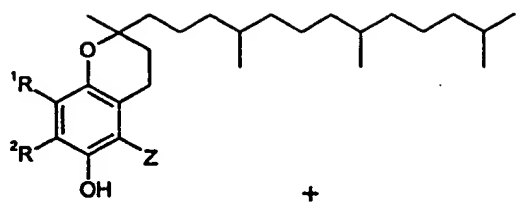
Alternatively, in relation to the compounds of formula II, particularly when Z is other than a group of the formula $(CH_2)_nXH$, reduction of the quinone to its hydroquinone form may facilitate an intramolecular cyclisation reaction via the hydroxy group present on the hydroquinone ring and subsequent elimination of the drug species. The resulting cyclic ether is non-cytotoxic.

Reaction scheme 1 below illustrates the preparation of a preferred bioreductive conjugate of formula II in which R^1, R^2 and Z are as hereinbefore defined. As will be seen bioreductive activation of the conjugate results in the formation of a cyclic ether which is an analogue of vitamin E and non-cytotoxic.

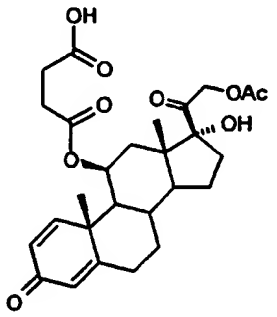
Scheme 1:


FeCl3/H2O
succinyl chloride
CH2Cl2
pyridine
then H2O
(Me)2NP(O)Cl2
DME
pyridine
bioreduction

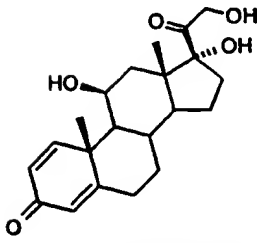
(Scheme 1 cont.)



+

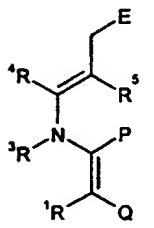


hydrolysis



PREDNISOLONE

Other preferred bioreductive conjugates in accordance with the invention include those compounds of formula III:



(III)

(wherein

P and Q together with the intervening ring carbon atoms form a quinone or indoloquinone ring, a nitroaromatic, N-oxide or diazoaromatic compound, itself optionally substituted by one or more halogen atoms, or by one or more groups selected from R, OR, SR, NHR, NR_2, CO_2R and CONHR;

R^1 represents a hydrogen or halogen atom, or a group R, OR, SR, NHR, NR_2, CO_2R or CONHR;

R^3, R^4 and R^5 independently represent hydrogen atoms or an alkyl or alkenyl group;

each group R independently represents a hydrogen atom, an alkyl or alkenyl group;

E represents the residue of a therapeutic agent to be delivered, optionally attached via a linking group L);

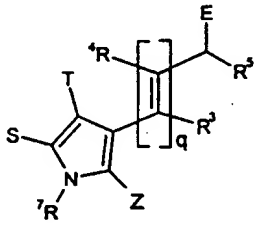
or a salt thereof.

Preferred compounds of formula III are those wherein P and Q together with the intervening ring carbon atoms form a quinone or indoloquinone ring; and

R^1, R^3, R^4 and R^5 each represent hydrogen atoms or methyl groups.

To act as "self-alkylating" systems, the electron-rich heteroatom present in the reduced form of the ring system of the compounds of formula III should preferably be no more than 6 bonds from the carbon atom linked to the therapeutic agent, E.

Other preferred bioreductive conjugates in accordance with the invention include the compounds of formula IV:



(IV)

(wherein

S and T together with the intervening ring carbon atoms form a quinone or iminoquinone ring, a nitroaromatic or N-oxide, e.g. an aromatic N-oxide, compound, itself optionally substituted by one or more halogen atoms, or by one or more groups selected from R, OR, SR, NHR, NR_2, CO_2R and CONHR;

Z represents an alkyl, alkenyl, aryl or aralkyl group optionally carrying at least one OH, SH, NH_2 or NHR^6 group in which R^6 is an alkyl group;

R^7 represents an alkyl group, preferably C_{1-2} alkyl;

R^3, R^4 and R^5 independently represent hydrogen atoms or an alkyl or alkenyl group;

each group R independently represents a hydrogen atom, an alkyl or alkenyl group;

q = 0, 1, 2 or 3, preferably 0 or 1;

E represents the residue of a therapeutic agent to be delivered, optionally attached via a linking group L);

or a salt thereof.

Preferred compounds of formula IV are those in which S and T together with the intervening ring carbon atoms form a quinone or N-oxide compound;

R^3, R^4 and R^5 each represent hydrogen atoms;

R^7 is methyl;

Z represents a group of formula $(CH_2)_nXH$ wherein X represents an oxygen or sulphur atom or, preferably, a group of formula NY in which Y represents a hydrogen atom or an alkyl group, and n = 0, 1, 2 or 3; and

q = 0 or 1.

In relation to the compounds of formula IV, alkylating activity may effectively be abolished following drug delivery by choosing as group Z a bulky group capable of providing steric hindrance. In such cases, Z is preferably a linear or, more preferably, branched, C_{4-20} alkyl or alkenyl group. Alternatively, such compounds may act as "self alkylating" systems in cases where Z represents a group of the formula $(CH_2)_nXH$.

In each of the compounds of general formulae II-IV above, the substituents R, R^1, R^2, R^3, R^4, R^5, R^6 and R^7may be selected to provide the conjugate with optimum redox potential, solubility, enzyme specificity etc.

As used herein, the term "heterocyclic group" is intended to define a carbocyclic group interrupted by at least one heteroatom selected from oxygen, sulphur and nitrogen.

Examples of preferred carbocyclic or heterocyclic rings include benzene, pyridine, pyrrole, furan, pyrazine, piperidine, piperazine, pyrrolidine, morpholine and thiomorpholine rings.

In each of the compounds of formulae II-IV, preferred halogen atoms are fluorine and chlorine.

In the bioreductive conjugates of the invention, any alkyl or alkenyl moiety, unless otherwise stated, may be straight-chained or branched and preferably contains from 1 to 8, more preferably 1 to 6, and especially preferably 1 to 4, carbon atoms. Aryl moieties, unless otherwise stated, preferably contain from 5 to 12 ring atoms and especially preferably comprise phenyl rings.

Preferred salts of the compounds of formulae I-IV are those which are suitable for administration to patients and are thus pharmaceutically or physiologically acceptable salts. Such salts may be formed with various inorganic and organic acids and include the ammonium, alkali and alkaline earth metal salts.

Reductases known to be involved in activation of bioreductive compounds include DT diaphorase, cytochrome P450, NADPH-dependent cytochrome P450 reductase and xanthine oxidase. The ease of reduction of any given bioreductive agent will depend upon its ability to act as a substrate for the intracellular reductases and the expression levels of such enzymes within the particular cell type. The choice of bioreductive compound for use in the invention will thus depend upon the type of enzymes present at

the target site. Indeed, it may be useful to determine the relative enzyme activities in the target tissues of individual patients before starting treatment.

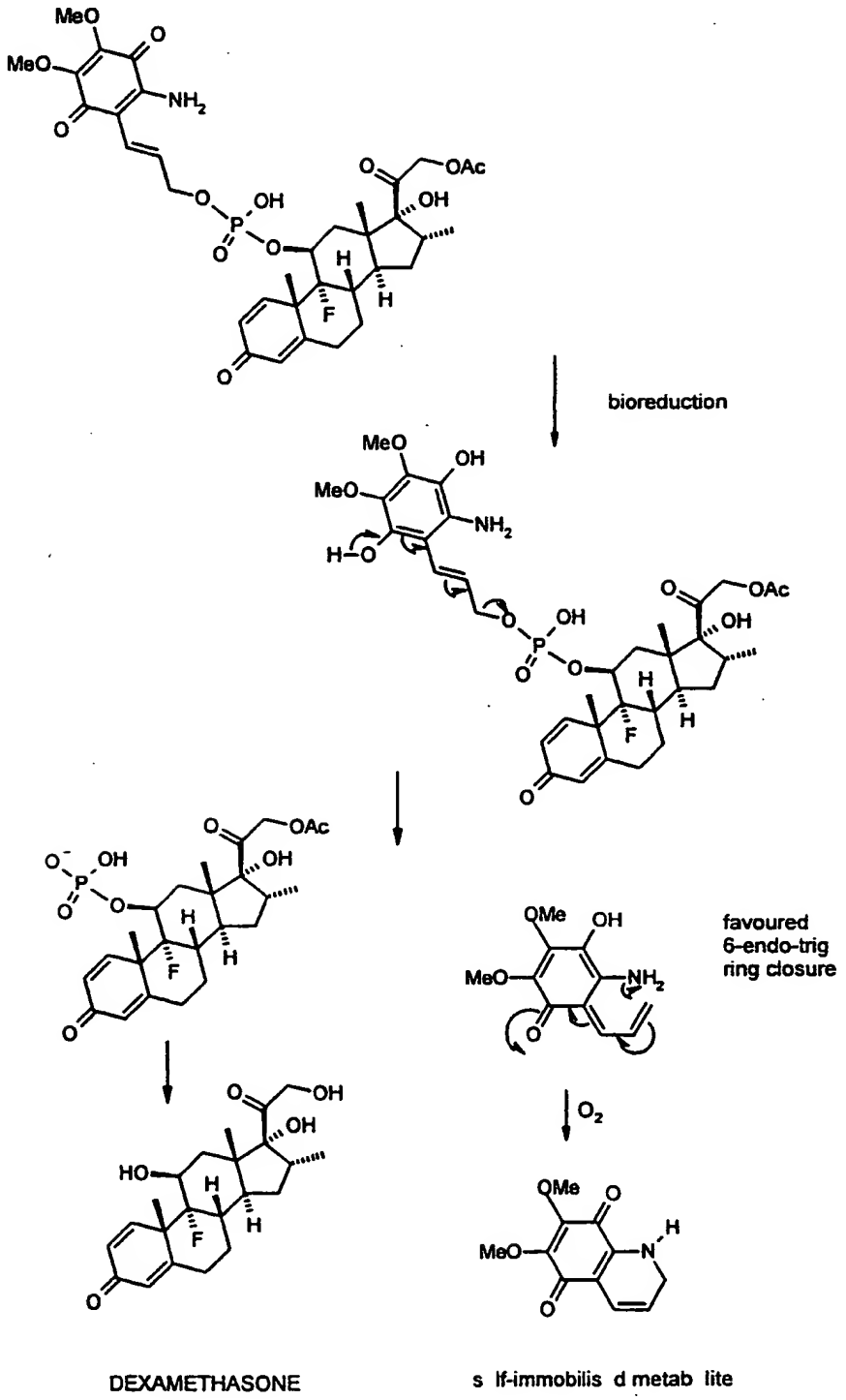
It is clearly desirable that the bioreductive conjugate should reach the target site intact. Since bioreduction of the conjugate is dependent upon the redox potential of the bioreductive moiety present, this may be selected such that this is less susceptible to reduction by ubiquitous systems such as NADH or NADPH, thereby increasing the chances that the conjugate will reach the target site still intact. In general, those bioreductive compounds having an optimal redox potential will be more selective in targeting of hypoxic cells and are thus preferred for use in the invention.

Examples of bioreductive compounds preferred for use in the invention include the quinones, naphthoquinones, indoloquinones and quinolino quinones and their derivatives. The electron deficient quinone nucleus in such compounds readily undergoes reduction *in vivo* to form the corresponding electron rich hydroquinone which in turn is capable of intramolecular rearrangement to release the drug. Particularly preferred quinones include the 1,4-benzoquinones and the naphthoquinones in which the quinone ring carries an optionally hydroxy or amino substituted alkenyl group, e.g. a propenyl group, and an adjacent nucleophilic moiety, e.g. an amino group. Indoloquinones are particularly good substrates for DT diaphorase, an enzyme commonly found in most tissues.

A particularly preferred bioreductive conjugate in accordance with the invention is shown in reaction scheme 2 given below in which the bioreductive moiety is a 1,4-benzoquinone and the therapeutic agent is dexamethasone, an anti-inflammatory agent which may be used in the treatment of rheumatoid arthritis.

Scheme 2:

Scheme 2:


bioreduction
favoured
6-endo-trig
ring closure
DEXAMETHASONE
s lf-immobilis d metab lite

The invention is considered to have utility in connection with the delivery of a wide range of therapeutic agents. The expressions "therapeutic agent" and "drug" are used interchangeably herein and are intended to define any atom, ion or molecule which *in vivo* is capable of producing an effect detectable by any chemical, physical or biological examination. A therapeutic agent will in general be any substance which may be administered to a human or non-human animal body to produce a desired, usually beneficial, effect and may be an agent having either a therapeutic or a prophylactic effect.

Examples of therapeutic agents suitable for use in accordance with the invention include agents in all of the major therapeutic areas including anti-infectives such as antibiotics and antiviral agents, analgesics, anaesthetics and anti-inflammatory agents. Anti-neoplastics, including known cytotoxic agents may also be used. The exact choice of therapeutic agent will naturally depend upon the desired therapeutic application.

Whilst it is envisaged that in general the therapeutic agent will itself be non-cytotoxic, the bioreductive carrier may be used to deliver cytotoxic agents, e.g. in anti-tumor treatment.

Examples of other therapeutic agents for use in accordance with the invention include agents administered to the human or animal body for diagnostic purposes, e.g. for use in radioimaging techniques. In this regard, a radiolabelled steroid may be linked to a non-cytotoxic bioreductive compound for use in the detection of hypoxic cells in tumor tissues.

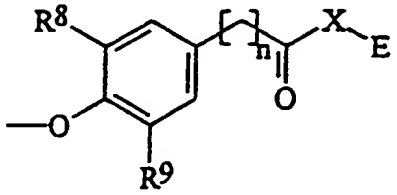
Methods for attaching bioreductive compounds to a therapeutic agent are within the level of skill in the art. In general, the conjugates in accordance with the invention can be prepared by linkage of a non-cytotoxic bioreductive moiety to at least one therapeutic agent. Linkage of the therapeutic agent to the bioreductive moiety may be effected through any reactive group and standard coupling techniques are known in the art. Preferred reaction conditions, e.g. temperature, solvents, etc. depend primarily on the particular reactants and can readily be determined by those skilled in the art. In general, any reactive groups present, e.g. amino, carboxy etc. will be protected during coupling of the bioreductive with the therapeutic agent, although it is possible to leave some groups unprotected. After coupling, the resulting compound may be purified, e.g. by chromatography.

The bioreductive moiety may be bonded directly to the therapeutic agent or may be bonded by a linker group, L. Linkage between the bioreductive and the therapeutic agent may be effected via any reactive group present in the bioreductive moiety, e.g. a primary amine, carboxylate, alcohol, thiolate, etc. Preferably, the bioreductive moiety is linked to the therapeutic agent via an ester, phosphate ester, ether, amine, thiol or thiol ester bond or any combination thereof.

The linker group serves to link the bioreductive moiety to at least one therapeutic agent. Besides filling this role as a linker, the linker group may be selected to yield a bioreductive conjugate having desired characteristics. For example, appropriate choice of a linker group may serve to enhance the resistance of the conjugate to non-bioreductive metabolism and/or enhance delivery of the drug molecule at the target site. It may also be possible to optimise the redox potential, enzyme or tissue specificity, or the solubility of the conjugate by attaching to or incorporating within the linker group appropriately selected moieties, e.g. groups which are tissue targeting. Thus, the ability to alter the nature of the linker group provides for the possibility of altering the physiochemical properties, e.g. solubility, and biological properties, e.g. biodistribution, of the bioreductive conjugate. The primary function of the linker is however to link together the bioreductive compound and the drug.

Linker groups L particularly suitable for use in the invention for those drugs having a free -OH or -SH group include the following which E represents the residue of a drug species:

and



(wherein n is an integer from 1 to 3;

X represents a sulphur or oxygen atom which may form part of the drug molecule E;

and R^8 and R^9 each independently represent F or Cl).

The bioreductive itself may be synthesised in accordance with conventional synthesis techniques. Techniques for the synthesis of quinones, in particular indoloquinones are described for example in J. Org. Chem. 50: 4276-4281 (1985).

Viewed from a further aspect the invention provides a process for the preparation of a bioreductive conjugate useful in the present invention comprising a non-cytotoxic bioreductive moiety with linked thereto at least one therapeutic agent, said process comprising linking at least one therapeutic agent to a non-cytotoxic bioreductive moiety.

There are believed to be many conditions which may benefit from the drug delivery system of the invention. These are primarily conditions associated with hypoxia and/or ischemia. Hypoxia is any state in which a physiologically inadequate amount of oxygen is available to, or utilized by, any given tissue or group of tissues within the body. Ischemia is any local diminution in the blood supply to any tissue in the body and may arise as a result of obstruction in the flow of arterial blood or vasoconstriction. In general, ischemia will ultimately lead to hypoxia.

In a clinical setting, tissues may become hypoxic and/or ischemic as a result of a number of different conditions in the body. Reduction of the blood supply to body tissues has the effect of inducing ischemia, for example in atherosclerosis, diabetes or following tissue or organ transplantation. Inflammatory or cancerous response may also lead to the tissue either physically or metabolically outgrowing its vascular supply, again leading to ischemia and/or hypoxia.

Non-limiting examples of conditions which may be treated using the bioreductive conjugates of the invention include inflammatory conditions, e.g. rheumatoid arthritis, and ther arthritic conditions such as osteoarthritis, diabetes, atherosclerosis, stroke, sepsis, Alzheimer's disease and other neurological diseases, cancer, kidney disease, digestive diseases and liver disease. Other conditions of interest include chronic periodontitis and ischemia following tissue transplantation.

The bioreductive conjugates of the invention may also find use in the treatment of a wide range of inflammatory conditions in which hypoxia and/or ischemia may be implicated, in particular in treating inflammatory conditions of the soft tissues. In the case of certain inflammatory conditions of the gastrointestinal tract, sections of the g.i. tract become hypoxic. Other inflammatory conditions which may be treated in accordance with the invention thus include gastrointestinal disorders such as Crohn's disease.

The compounds of the invention may also be used in the treatment of muscular disorders associated with hypoxia and/or ischemia.

It is believed that many known drugs could have enhanced therapeutic effects if selectively delivered to ischemic/hypoxic tissue. For example, following a cerebral attack, cerebral perfusion is reduced and the brain suffers an inflammatory response. The linkage of a vasodilator, such as a nitric oxide generator, or an anti-inflammatory agent, such as a steroid, to a bioreductive agent would thus serve to enhance the therapeutic index of the drug.

Rheumatoid arthritis is known to be associated with chronic synovial inflammation and poor perfusion of the synovial tissues. However, we have now discovered that in patients suffering from rheumatoid arthritis the synovial tissues are in many cases profoundly hypoxic (pO_2 < 12 mm Hg). We have also found that such tissues contain high levels of reductases. Whilst not wishing to be bound by theoretical considerations, it is believed that there are pockets in the synovium which are hypoxic and that it is the

hypoxic cells in the synovium which are primarily responsible for the inflammation associated with rheumatoid arthritis. Linkage of an anti-inflammatory agent, such as a non-steroidal anti-inflammatory agent, e.g. dexamethasone, a steroid or a nitric oxide inhibitor would thus serve to greatly increase the therapeutic index of the active agent in the treatment of rheumatoid arthritis, whilst at the same time reducing the risk of systemic side effects. The weak acidic based NSAIDs which undergo ion-trapping in acidotic tissue are considered particularly suitable.

Following transplantation and tissue rejection, both ischemia and an immunological-inflammatory response may contribute to tissue hypoxia. Again, such conditions may thus be treated using a conjugate of the invention in which a bioreductive moiety is linked to a vasodilator or to an anti-inflammatory or immunological suppressant.

Many of the basic complications of diabetes are believed to owe their basic pathology to hypoxia. Indeed, in many cases diabetics show accelerated atherosclerosis. The present invention may thus be used in the treatment of diabetes by linking a drug, such as a phosphodiesterase inhibitor, to a non-cytotoxic bioreductive moiety.

Hypoxic tissues are also believed to be present in chronic periodontitis, a condition associated with severe inflammation of the periodontium. Linkage of an antibiotic or other drug known for treating periodontitis, e.g. a metalloproteinase inhibitor, to a bioreductive may thus be beneficial in treating this condition.

An example of an agent which may be linked to a non-cytotoxic bioreductive compound for use in treating diabetes is dipyridamole.

Viewed from yet a further aspect, the invention provides a prodrug activating agent comprising a bioreductive conjugate as hereinbefore defined for use in a method of targeting a therapeutic agent to a specific tissue site within the body, in particular to a site of hypoxia and/or ischemia, e.g. in the treatment of rheumatoid arthritis or other arthritic conditions, diabetes, atherosclerosis, stroke, sepsis, Alzheimer's disease and

other neurological disorders, cancer, kidney disease, digestive diseases, liver disease, chronic periodontitis or ischemia following tissue transplantati n.

In a preferred embodiment the invention provides a prodrug activating agent comprising a bioreductive conjugate comprising a non-cytotoxic bioreductive moiety linked to an anti-inflammatory agent for use in the treatment of rheumatoid arthritis.

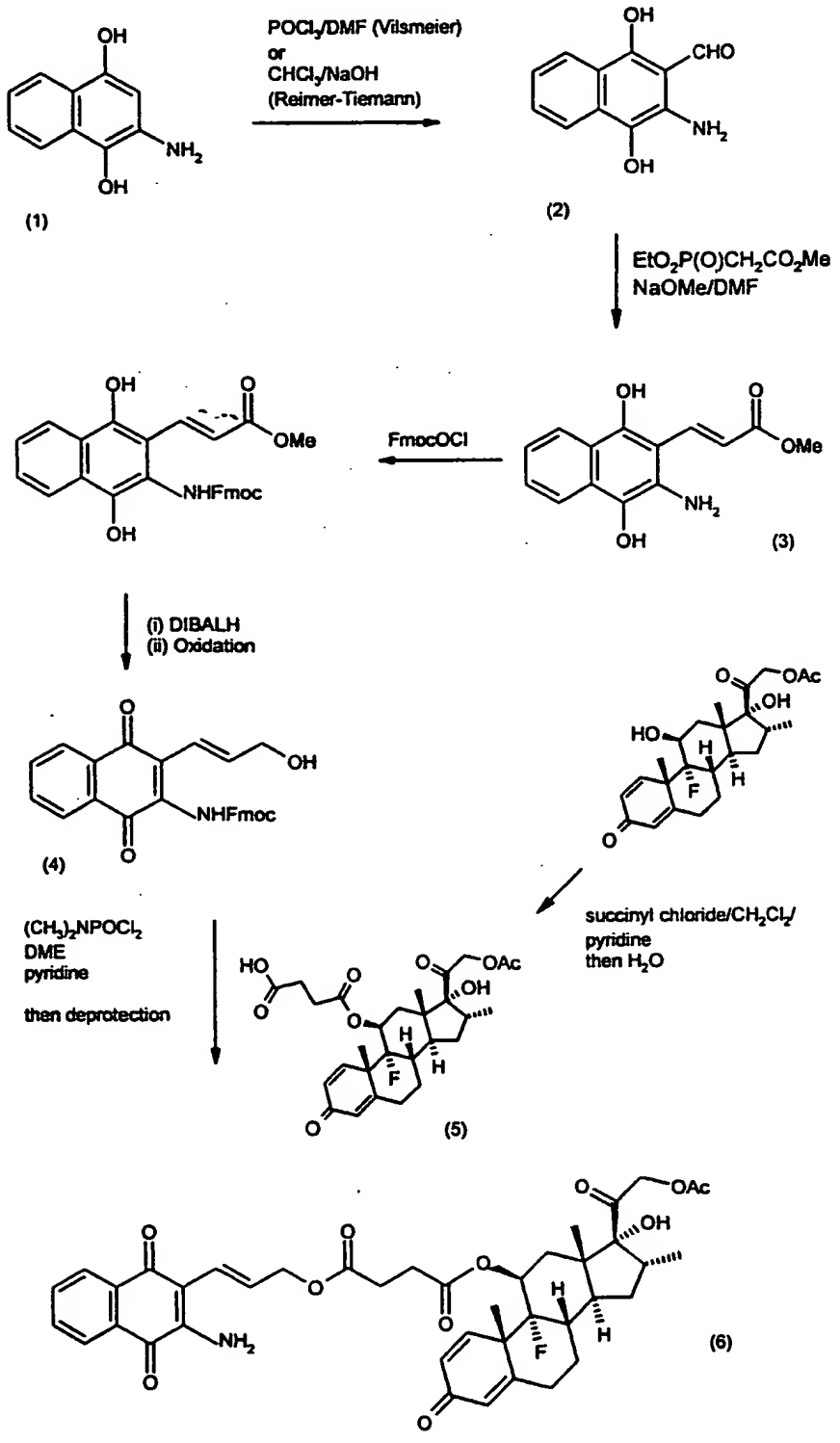
Viewed from yet a further aspect the invention provides the use of a prodrug activating agent comprising a bioreductive conjugate as hereinbefore defined in the manufacture of a medicament for use as a targeting agent, in particular as an agent capable of targeting a site of hypoxia and/or ischemia within the body, e.g. in the treatment of rheumatoid arthritis and other arthritic conditions, diabetes, atherosclerosis, stroke, sepsis, Alzheimer's disease and other neurological disorders, cancer, kidney disease, digestive diseases, liver disease, chronic periodontitis or ischemia following tissue transplantation.

In another aspect the invention provides a method of targeting hypoxic and/or ischemic tissues in the human or non-human, preferably mammalian, body comprising administering to said body aprodrug activating agent comprising a bioreductive conjugate as hereinbefore defined. In particular, the invention provides a method of treating or preventing rheumatoid arthritis and other arthritic conditions, diabetes, atherosclerosis, stroke, sepsis, Alzheimer's disease and other neurological disorders, cancer, kidney disease, digestive diseases, liver disease, chronic periodontitis or ischemia following tissue transplantation, said method comprising administering to a human or non-human animal body in need thereof an effective amount of a bioreductive conjugate as hereinbefore defined.

Viewed from yet a further aspect the invention provides a pharmaceutical composition comprising a prodrug activating agent comprising a bioreductive conjugate in accordance with the invention or a pharmaceutically acceptable salt therof, together with at least one pharmaceutical carrier or excipient.

The present invention will now be further illustrated by way of the following non-limiting Examples and with reference to accompanying Figure 9 which shows the product profile obtained on the reduction of the asprin-bioreductive conjugate of Example 5 by the $(CH_3)_2C^{\bullet}OH$ radical.

Example A - Synthesis of "self-alkylating" bioreductive delivery system.



Step 1 - N,N-dimethyl formamide (2 equivs) and $POCl_3$ are stirred together. The resulting solution is then added to a solution of the protected amino-dihydro-napthoquinone (1 equiv) in 1,2-dichloroethane and heated under reflux for about 1½ hours. The resulting solution is then cooled and NaOAc (1M, 100 mL/g quinone) is added with stirring over 2½ hours. The solution is then extracted with EtOAc, dried and evaporated. The resulting product (2) is then purified by chromatography on silica.

Step 2 - triethylphosphonoacetate (10.92 mmol) is stirred into dimethylformamide (80 ml). NaOMe (11 mmol) is then added and the solution is stirred for ½ hour. Product (2) (4.27 mmol) dissolved in dimethylformamide (20 ml) is added stepwise and stirring is continued for a further 2 hours. The mixture is then diluted with ethyl acetate (300 mL), washed with aqueous sodium hydrogen carbonate (6 x 100 mL), dried, evaporated *in vacuo* and the product (3) is recrystallised from ethyl acetate.

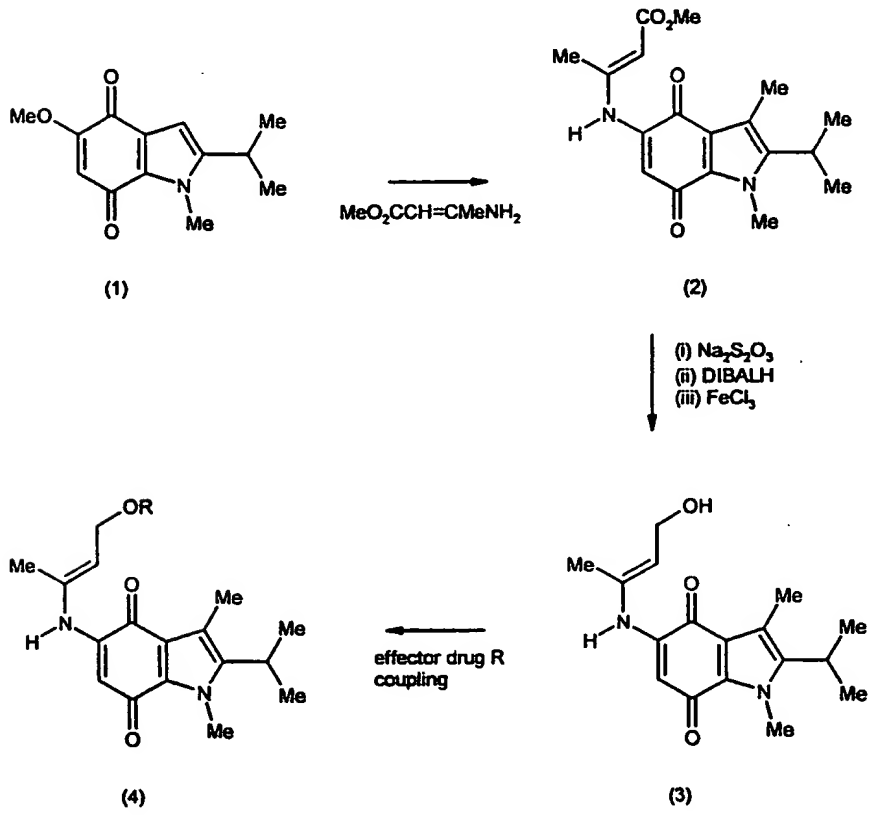
Step 3 - Product (3) (1.21 mmol) is dissolved in anhydrous CH_2Cl_2 (90 mL) and diisobutylaluminium hydride (16.3 mL, 1.5M in toluene) is added dropwise at -50°C. The mixture is then stirred for 3½ hours at -30°C and $FeCl_3$ (1.0M dissolved in 0.1M HCl, 27mL) is added keeping the temperature below 0°C. Stirring is continued for a further ½ hour at 0°C followed by filtration. The resulting product is extracted with $CHCl_3$ (4 x 75 mL), washed with brine (50 mL), dried and evaporated *in vacuo*. Product (4) is recrystallised in ethanol.

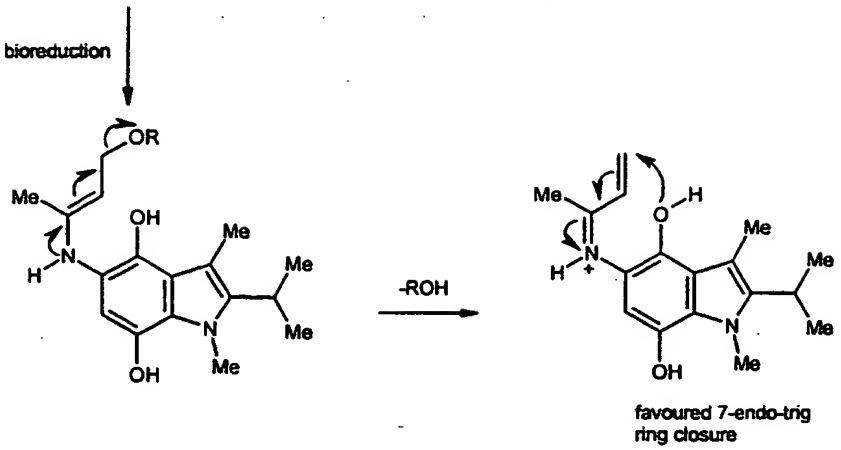
Step 4 - prednisolene 21-acetate (1 equiv) is dissolved in dry CH_2Cl_2 (50 mL) and dry pyridine (10 mL) is added under an atmosphere of nitrogen. The solution is then stirred under reflux for 2 hours together with succinyl chloride (1.1 equivs). This is then cooled and washed with dilute HCl (0.1M, 20 mL) followed by H_2O (3 x 30 mL), dried and evaporated *in vacuo*. Product (5) is purified by chromatography on silica.

Step 5 - pyridine (6 mmol), N,N'-dimethylphosphoramidic dichloride (3 mmol) and product (4) (4 mmol) are added to a solution of product (5) (2 mmol) in 1,2-

dimethoxyethane (10 mL) at 0°C. The resulting solution is stirred at ambient temperature under an atmosphere of argon for 16 hours. This is then poured into ice cold 1N HCl (40 mL) and extracted with CH_2Cl_2 (4 x 30 mL). The combined extracts are dried with $MgSO_4$, filtered and concentrated. The residue is purified by column chromatography on silica gel to give the final product (6).

Example B - Synthesis of "self-alkylating" bioreductive delivery system.





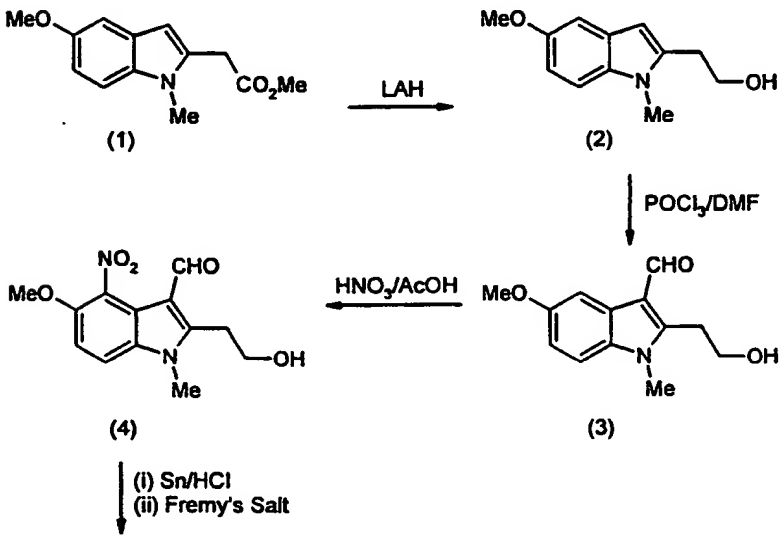
Step 1 - Compound (1) (10 mmol) (see Naylor et al., 2-Cyclopropyl Indoloquinones and their Analogues As Bioreductively-Activated Antitumor Agents: Structure-Activity *in vitro* and Efficacy *in vivo*, J. Med. Chem.: 40 (15), 1997) is dissolved in DMF (10 mL) and methyl 3-aminocrotonate (50 mmol) is added. The reaction mixture is stirred at ambient temperature for 18 hours and then evaporated *in vacuo* and the residue purified on silica to give product (2).

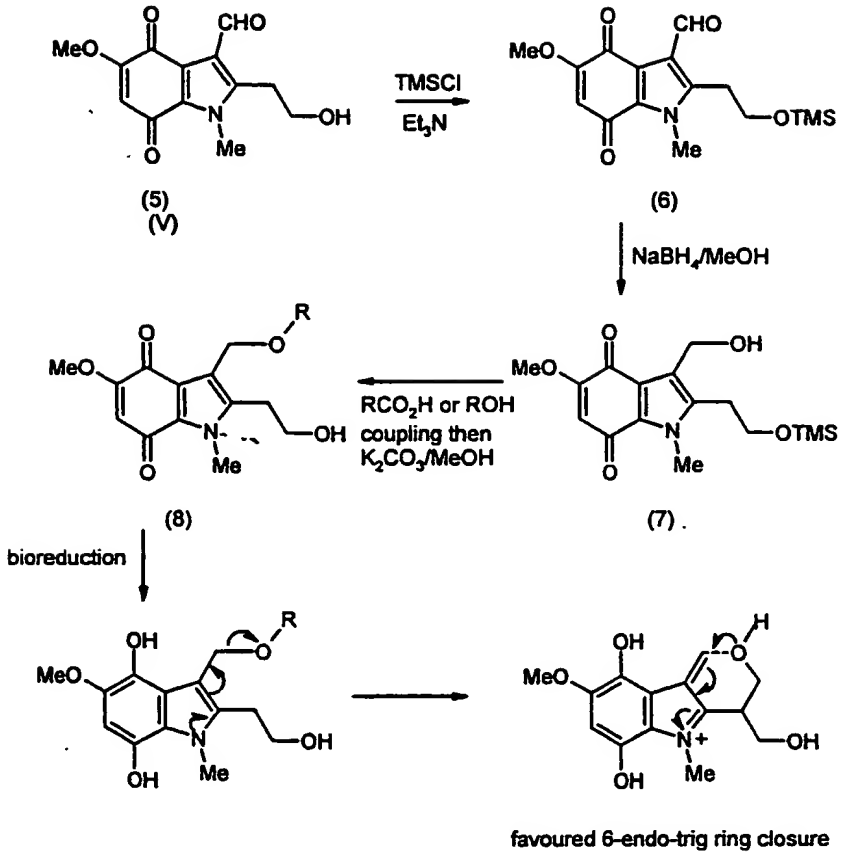
Step 2 - the aminocrotonate derivative (2) (10 mmol) is dissolved in $CHCl_3$ (300 mL) and EtOH (110 mL) and a solution of $Na_2S_2O_4$ (120 mmol) in H_2O (130 mL) added. The solution is stirred at ambient temperature for ½ hour and the organic layer separated, washed with saturated NaCl (500 mL), dried and evaporated. The crude hydroquinone is then dissolved in anhydrous CH_2Cl_2 (300 mL) under argon, cooled to -30°C and DIBAL-H (50 mL of a 1.5M solution in toluene) added dropwise such that the solution temperature remains below -30°C. The solution is then allowed to reach 0°C and stirred for 2½ hours at this temperature, and a solution of solution of $FeCl_3$ (90 mL, 1.0M (0.1M HCl)) added. The solution is stirred for 10 min at 0°C and then $CHCl_3$ (500 mL) and H_2O (500 mL) added. The aqueous layer is extracted with $CHCl_3$ (5 x 250 mL) then EtOAc (5 x 200 mL) and the combined organic phases washed with

saturated NaCl (500 mL), dried and evaporated. The residue is purified on silica and recrystallized from EtOAc to give product (3) as a purple/red solid.

Step 3 - the indoloquinone (3) (10 mmol) is dissolved in THF (25 mL) and added to a solution (THF, 25 mL) of the drug carboxylic acid or phenol to be coupled (1.5 equivs), triphenylphosphine (20 mmol) and diethylazodi-carboxylate (20 mmol). The solution is then stirred overnight at 50°C, the solvent evaporated and the residual final product (4) is purified on silica.

Example C - Synthesis of "self-alkylating" bioreductive delivery system.





Step 1 - Methyl 5-Methoxy-1-methylindole-2-acetate (10 mmol) is dissolved in anhydrous THF (250 mL) and $LiAlH_4$ (100 mL of a 1.0M solution in THF) added dropwise at ambient temperature and under argon. The solution is then stirred for 1 hour at 30°C and then EtOAc (250 mL) added, followed by the gradual addition of H_2O (150 mL). The solution is washed with HCl (0.1M, 250 mL) and saturated NaCl (250 mL), dried and evaporated. The residue is purified by flash column chromatography on silica and then recrystallized to give product (2).

Step 2 - DMF (100 mmol) and $POCl_3$ (25 mmol) are stirred at -5°C for ½ hour and then a solution of (2) (10 mmol in 30 mL DMF) is added slowly, maintaining the

temperature at about 0°C, and then warmed to 40°C and stirred for 1 hour. Ice/water (100 mL) is then added, followed by NaOH (37%, 50 mL) and the solution extracted into EtOAc, evaporated and the carboxaldehyde (3) purified by recrystallization from an EtOAc/hexane mixture.

Step 3 - to a solution of (3) (10 mmol) in AcOH (50 mL) cooled to 5°C, is added dropwise a cold (0°C) mixture of fuming HNO_3 (10 mL) in AcOH (30 mL). The solution is stirred for 1 hour while allowing to reach ambient temperature, and then poured onto 100g of crushed ice. After 15 minutes stirring the resulting yellow solid is collected by suction filtration. The dried residue is purified on silica to give product (4) as a yellow solid.

Step 4 - to a suspension of (4) (10 mmol) in EtOH (180 mL) is added tin powder (40 mmol) and HCl (3.0M, 70 mL) and the solution stirred at ambient temperature for 1 hour. The solution is then decanted from the excess tin and neutralized with saturated $NaHCO_3$(aq.). The resulting suspension is then added to an equal volume of H_2O and extracted with $CHCl_3$ (5 x 50 mL) and then EtOAc (5 x 50 mL) and the combined extracts evaporated. The residual 4-aminoindole derivative is purified on silica and used immediately in the next step by dissolving in Me_2CO (250 mL) and adding a solution of potassium nitrosodisulfonate ($(KSO_3)_2NO$, Fremy's salt, 30 mmol)) in NaH_2PO_4/Na_2HPO_4 buffer (250 mL, 0.3M, pH 6.0) and the solution stirred at ambient temperature for 1 hour. The Me_2CO is removed *in vacuo* and the resulting orange precipitate collected by suction filtration, washed with H_2O and drifed in avacuum oven at 45°C to afford product (5) as an orange solid which is recrystallized from EtOAc.

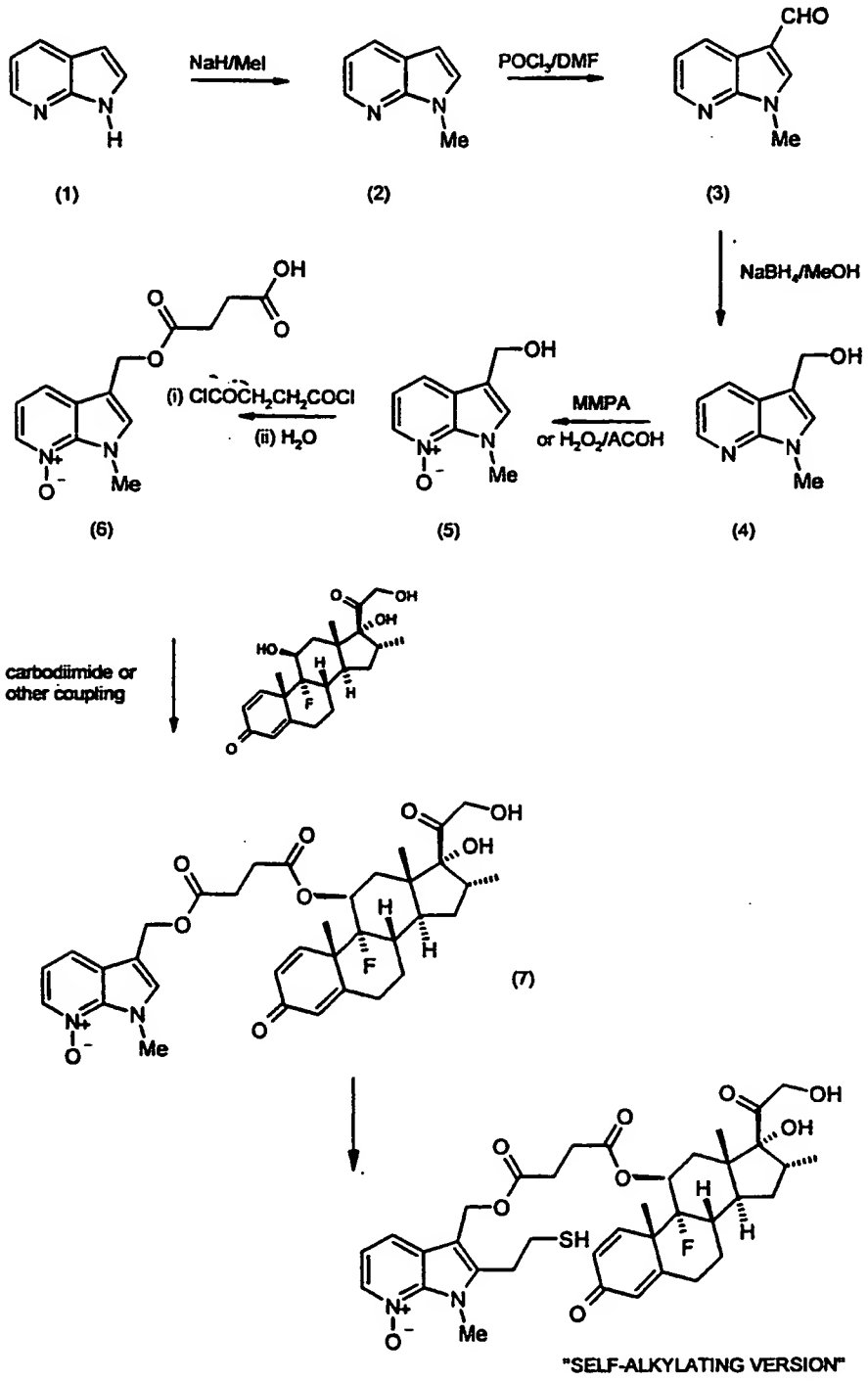
Step 5 - indoloquinone (5) (10 mmol) is dissolved in THF (100 mL) together with Et_3N (10 mmol) and trimethylchlorosilane (1.1 mmol) added. The solution is stirred at ambient temperature for 8 hours, evaporated and purified on silica to give product (6).

Step 6 - the protected indoloquinone (6) (10 mmol) is dissolved in anhydrous nitrogen degassed MeOH (200 mL) and $NaBH_4$ (30 mmol) added. The solution is degassed with

argon and stirred for 5 min under argon and then aerated and diluted with EtOAc (700 mL) and washed with H_2O (2 x 250 mL) and then saturated NaCl (100 mL). The dried organic solution is condensed to give the indoloquinone (7) as an orange solid after silica column and/or recrystallization from EtOAc.

Step 7 - the 3-(hydroxymethyl) indoloquinone (7) is dissolved in THF (50 mL) together with tripphenylphosphine (20 mmol) and deithylazodi-carboxylate (20 mmol) and the desired drug carboxylic acid or phenol (RCO_2H or ROH where R is a drug species, 1.5 to 5 equivs) added. The solution is then stirred overnight at 50°C, the solvent evaporated and the residue redissolved in EtOAc. The solution is then washed with HC1 (1.0M, 50 mL) and H_2O (50 mL), dried and evaporated. The product is purified on silica and deprotected by dissolving in anhydrous MeOH together with K_2CO_3 (10 mmol) at 0°C and stirring for 45 min. The final product (8) is then purified on silica and recrystallized from EtOAc.

Example D - Synthesis of "self-alkylating" bioreductive delivery system.



Step 1 - 7-Azaindole (Sigma-Aldrich, 10 mmol) is added gradually with stirring to a suspension of NaH (11 mmol) in THF (30 mL). After 15 minutes, methyl iodine (10 mmol) is added and the solution stirred at ambient temperature for 1 hour. The solution is cooled to -5°C and H_2O (30 mL) added gradually, followed by EtOAc (50 mL). The aqueous layer is then further extracted with EtOAc (3 x 50 mL), washed and saturated $NaHCO_3$, saturated NaCl, dried and evaporated. The residue is purified on silica to give product (2).

Step 2 - DMF (100 mmol) and $POCl_3$ (25 mmol) are stirred at -5°C for ½ hour and then a solution of (2) (10 mmol in 30 mL DMF) is added slowly, maintaining the temperature at about 0°C, and then warmed to 40°C and stirred for 1 hour. Ice/water (100 mL) is then added, followed by NaOH (37%, 50 mL) and the solution extracted into EtOAc, evaporated and the carboxaldehyde (3) purified by recrystallization from an EtOAc/hexane mixture.

Step 3 - the 3-formyl-7-azaindole (3) (10 mmol) is dissolved in anhydrous nitrogen degassed MeOH (200 mL) and $NaBH_4$ (30 mmol) added. The solution is degassed with argon and stirred for 5 min under argon and then aerated and diluted with EtOAc (700 mL) and washed with H_2O (2 x 250 mL) and then saturated NaCl (100 mL). The dried organic solution is condensed to give the 3-hydroxymethyl derivative (4) after silica column chromatography.

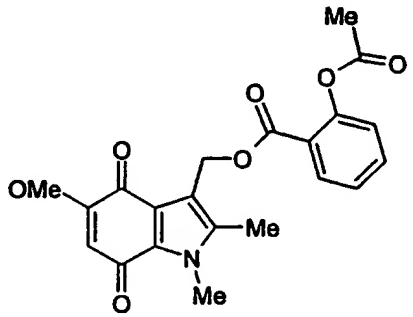
Step 4 - product (4) (10 mmol) is dissolved in KOH (0.5M, aq., 100 mL). Caro's acid (potassium peroxymonsulphate, Oxone, $2KHSO_5 . KHSO_4 . K_2SO_4$, 10 mmol) is added slowly with stirring and the solution stirred for 12 hours. The solution is neutralised with phosphoric acid, evaporated and the residual salt extracted and purified on silica to afford (5).

Step 5 - the 3-(hydroxymethyl) indole (5) (10 mmol) is dissolved in THF (50 mL) together with pyridine (5 mL) and succinylchloride (10 mmol) added with stirring.

After 1 hour H_2O (50 mL) is added and the solution stirred for 1½ hours and 2.0M HCl (50 mL) added. After a further 1½ hours the solutin is extracted with Et_2O (3 x 100 mL), dried and evaporated. The acid (6) is purified on silica.

Step 6 - the azaindole-N-oxide carboxylic acid (6) (10 mmol) is dissolved in THF (25 mL) and added to a solution (THF, 25 mL) of the protected steroid (1.5 equivs), triphenylphosphine (20 mmol) and diethylazodicarboxylate (20 mmol). The solution is then stirred overnight at 50°C, the solvent evaporated and the residue redissolved in EtOAc. The solution is washed with HCl (1.0M. 50 mL) and saturated $NaHCO_3$ (aq., 50 mL), dried and evaporated. The final product (7) is purified on silica.

Example E - Preparation of 3-(2-Acetoxybenzoyloxy) methyl-1,2-dimethyl-5-methoxyindole-4, 7-dione: Asprin-Bioreductive Conjugate



3-Hydroxymethyl-5-methoxy-1, 2-dimethylindole-4, 7-dione (0.235g, 1.0 mmol) was dissolved in dichloromethane (anhydrous, 25 mL) together with pyridine (2.5 mL). 2-Acetylsalicyloyl chloride (0.237g, 1.2 mmol) was then added and the solution heated under reflux for 1½ hours, cooled and ethyl acetate (100 mL) added. The solution was washed with HCl (0.1 M, 100 mL) and then saturated NaCl (100 mL), dried and evaporated. The residue was purified on silica gel, eluting with ethyl acetate to afford the title compound as a yellow solid (275 mg, yield: 69.3%) which was recrystallised from ethyl acetate, mp 159-161°C.

^{1}H-NMR ($CDCl_3$) σ 2.27 (s, 3H), 2.31 (s, 3H), 3.81 (s, 3H), 3.90 (s, 3H), 5.47 (s, 2H), 5.63 (s, 1H), 7.01-7.53 (m, 3H) and 7.99 (dd, J = 1.4 and 8.1 Hz, 1H) ppm.

Analysis: Found : C 63.81, H 4.81, N 3.71
Calculated : C 63.47, H 4.82, N 3.52%

Example F

Pharmacokinetics of the indoloquinone-acetal salicylic acid conjugate of Example 5 were studied as follows:

PROTOCOL

Three groups of male Winstar albino rats (n=5) received sterile air dorsally (day 1). After two days a further 20 ml sterile air were administered. On day 5, 2 ml of a 1% carrageenin in sterile saline was injected directly into the air pouch. Animals were housed in metabolic cages.

100 mg of the indoloquinone-asprin conjugate of Example 5 were suspended in ethanol (2 ml). 50 mg acetyl salicylic acid was dissolved in 2 ml ethanol. 2 ml ethanol was used as a control. 18 ml sterile water were added to each sample.

On day 9, each animal was injected with 4 ml of solution as follows:

Group A - 20 mg indoloquinone-asprin conjugate

Group B - 10 mg acetyl salicylic acid

Group C - ethanol (control)

The animals were then returned to their cages for periods of either 2 (nos. 1, 2 and 3 from each group) or 4 hours (nos. 4 and 5 from each group). After this time the animals were anaesthetised and blood and exudate collected. Available urine was also collected.

RESULTS:

Analysis of the collected samples by HPLC showed that the bioreductive-acetyl salicylic acid conjugate had been cleaved to liberate acetyl salicylic acid.

Example G

The reduction initiated release of asprin from the indoloquinone-acetyl salicylic acid conjugate of Example 5 was investigated by product analysis (HPLC) following Υ-radiolysis of N_2O-saturated solutions containing the quinone (100 μM) and 2-propanol (8.3M, 50%, v/v) at pH 7.4.

The radiation chemical yield (G) of the $(CH_3)_2C^{\bullet}OH$ radical in N_2O-saturated 2-propanol/water mixtures was determined by ferricyanide reduction to be $G((CH_3)_2C^{\bullet}OH) = 0.67 \pm 0.02$ μmol J^{-1} in 2-propanol/water (50%, v/v) and 0.72 ± 0.03 μmol J^{-1} in 1 M 2-propanol respectively. Figure 9 shows the product profile obtained on the reduction of the quinone by the $(CH_3)_2C^{\bullet}OH$ radical. Loss of the parent quinone ($G(-Q) = 1.63 \pm 0.01$ μmol J^{-1}) parallel the formation of the asprin leaving group (LG) with $G(LG) = 1.40 \pm 0.15$ μmol J^{-1}.

The two remaining major peaks in Figure 9 were derived from the reaction of the resultant iminium derivative with water to generate (a) and with the 2-propanol to generate the isopropyl ether (b). Both of these quinones are generated by autoxidation of their respective hydroquinones following the unavoidable introduction of oxygen during HPLC sampling:

$$QH_2 + O_2 - Q + H_2O_2$$

As expected, the relative yields of (a) and (b) were dependent on the alcohol concentration, with the alkylation product virtually disappearing when radiolysis was performed in 1M 2-propanol.

Steady-state ϒ-radiolysis

Indolequinone solutions were saturated with N_2O gas in gas-tight vials before irradiation in a ^{60}Co source. An absorbed dose of 1 Gy = 0.67 μM $(CH_3)_2C^{\bullet}OH$ radicals in N_2O-saturated 2-propanol/water (50%, v/v). A dose rate of 6-6.5 Gy min^{-1} was used, as determined by Fricke dosimetry and radiation chemical yields were corrected for the absorbed dose in the various alcohol-water mixtures employed.

High performance liquid chromatography (HPLC)

Product analysis following

ϒ-radiolysis was performed by gradient HPLC separation on a 100 mm x 4.6 mm base-deactivated reverse-phase column (Hichrom RPB, Hichrom, Reading, U.K.). The eluents were (A) : KH_2PO_4 (5 mM), H_3PO_4 (5 mM), (B) : CH_3CN/H_2O (3:1, v/v), with a flow rate of 2 cm^3 min^{-1}. One of two lineare gradients was used for each compound: (1) 35-80% B in 8 min, or (2) 20-50% B in 5 min. Detection was at 232 nm using a Waters 486 detector (Watford, U.K.) and concentrations were determined from peak areas using Waters Maxima software.

Example H - Formulation

A compositon suitable for use in the treatment of rheumatoid arthritis is produced using the following ingredients:

dexamethasone	5 mg
starch	45 mg
microcrystalline cellulose	35 mg
polyvinylpyrrolidone (as 10% solution in water)	4 mg
sodium carboxymethyl starch	4.5 mg
magnesium stearate	0.5 mg
talc	1 mg
total	95 mg

The active ingredient, starch and cellulose are sieved and mixed thoroughly. The aqueous solution containing polyvinylpyrrolidone is mixed with the resulting powder and the mixture is then passed through a sieve. The resulting granules are dried and sieved again. The sodium carboxymethyl starch, magnesium stearate and talc are sieved and then added to the granules which, after mixing, are compressed on a tablet machine to yield tablets weighing 95 mg.

One tablet taken daily is suitable for the treatment of patients suffering from rheumatoid arthritis.

HAEMATOPOEITIC STEM CELLS (HSCs)

By way of background information, monocytes and their differentiated derivatives, macrophages are derived from a reservoir of embryonic cells, called HSCs which are capable of giving rise to a variety of distinct cell types. HSCs, in mammals, are found within the fetal liver, spleen and bone marrow but after birth and throughout adult life,

they are normally found only in the bone marrow. HSCs differentiate into various cell lineages under the influence of microenvironmental factors such as cell-to-cell interactions and the presence of soluble cell cytokines.

Four major cell lineages arise from the HSCs. These include: Erythroid (Erythrocytes); Megakaryocytic (platelets); Myeloid (granulocytes and mononuclear phagocytes); and Lymphoid (lymphocytes). In particular, the myeloid and lymphoid lineages are critical to the functioning of the immune system.

Myelopoiesis commences in the liver of the human foetus at about six weeks of gestation. Studies in which colonies have been grown *in vitro* from individual stem cells have shown that the first progenitor cell derived from HSCs is the colony forming unit (CFU) which can give rise to Granulocytes, Erythrocytes, Monocytes and Megakaryocytes (CFU-GEMM).

Maturation of these cells occurs under the influence of a network of tissue specific protein regulators which have been given a variety of names including growth factors, cytokines and interleukins. In the main, there is no functional or structural characteristic that distinguishes the different classes of growth factors. Most factors appear to be capable of stimulating multiple biological responses that depend critically on the differentiation state of their target cells. For example, one of the haemopoietic growth factors, granulocyte colony stimulating factor (G-CSF) stimulates proliferation of immature bone marrow cells as well as activating bacterial killing by mature neutrophils. Erthropoietin (EPO) and thrombopoietin (TPO) are structurally similiar cytokines and support respectively, the proliferation and differentiation for erythroid and megakaryocytic lineages as well as more primitive progenitors (Gotoh *et al* 1997 Ann Hematol 75: 207-213). TPO initiates its biologic effects by binding to the Mpl receptor, which is a member of the haematopoietic receptor family (Broudy *et al* 1997 Blood 89: 1896-1904). HOXB4 has been shown to be an important regulator of very early but not late haematopoietic cell proliferation (Sauvageau *et al* 1995 Genes Dev 9: 1753-1765). The soluble Kit ligand proteins (Kls) act as a ligand for the transmembrane

tyrosine kinase receptor C-kit and stimulate mast cell and erythroid progenitors (91EP-810609). The interleukins, include IL-1, Il-2, IL-3, IL-4, IL-5, Il-6 and IL-12, which are capable of activating HSCs (see "Molecular biology and Biotechnology Ed RA Meyers 1995 VCH Publishers Inc p 392-397).

These mediators, which are important in the positive regulation of haemopoiesis, are derived mainly from stromal cells in the bone marrow, but they are also produced by mature forms of differentiated myeloid and lymphoid cells. There are a number of successful growth factor combinations in use but combinations of IL-3 and IL-6 with or without other factors such as stem cell factor (SCF) active on primitive cells have achieved the best results (Bodine *et al* 1989 Proc Natl Acad Sci 86: 8897-8901; Luskey *et al* 1992 Blood 80: 396-402; Fraser *et al* 1990 Blood 76: 1071-1076). Other cytokines (such as TGFβ) may downregulate haemapoiesis.

The CFU-GM cell is the precursor of both neutrophils and mononuclear phagocytes. As the CFU-GM differentiates along the neutrophil pathway, several distinct morphological stages are seen. Myeloblast develop into promyelocytes and myelocytes, which mature and are released into the circulation as neutrophils. The one-way differentiation of cells from the CFU-GM into mature neutrophils is probably the result of acquiring specific growth/differentiation factor receptors at different stages of development.

Surface differentiation markers disappear or appear on the cells as they develop into granulocytes. For example, MHC class II molecules and CD38 are expressed on the CFU-GM but not on mature neutrophils. Other surface molecules acquired during the differentiation process include CD13, CD14 at low density, CD15, the β_1 integrin, VLA-4, the β_2 integrins CD11a, b and c associated with CD18 β_2 chains, complement receptors and CD16 Fcγ receptors.

CFU-GMs taking the monocyte pathway give rise initially to profilerating monoblasts. These differentiate into promonocytes and finally into mature circulating monocytes.

Circulating monocytes are thought to be a replacement pool for tissue-resident macrophages. The different forms of macrophages comprise the reticulo-endothelial system.

Like mature neutrophils, mature monocytes and macrophages lose CD34. However, unlike neutrophils, they continue to express significant levels of MHC class II molecules. These molecules are clearly important for the presentation of antigen to T cells. Monocytes also acquire many of the same surface molecules as mature neutrophils.

In addition to macrophages, most of the classical antigen-presenting cells (APCs) which include the follicular dentritic cells, Langerhans' cells and interdigitating cells are present at birth. While their origin is still unclear, it is likely that most are derived from bone-marrow stem cells. One possibility is that they are derived from the same CFU-GEMM precursor cell Morphological, cytochemical and functional differences would then be due to local microenvironmental influences such as cytokines. Alternatively, APCs could be derived from different stem cells and represent separate lineages of differentiation.

In the first stage of differentiation into colony forming cells (such as CFU-GEMM) the HSCs express CD33 and CD34. Thus, HSCs can usually be characterised by the presence of the cell glycoprotein CD34 (and possibly CD33) at the cell surface.

In the next stage of differentiation to cells of the erythroid, myelomonocytic and megakaryotic lineages, the vital burst forming units-erythroid (BFUE) cells of the erythroid lineage carry antigens CD33 and CD34 but these antigens are lost in later differentiation. The myelomonocytic lineage which includes CFU-GM cells carry CD33 but not CD34 and this CD33 is subsequently lost. The megakaryotic lineage leads initially to CFU Mega cells which carry CD34 which is also subsequently lost.

A further significant system of antigens on HSC and other cells is the MHC (major histocompatibility complex) Class II group. It has been found that the majority of HSC carry an antigen termed DR and on differentiation express an antigen termed DP and then a further antigen termed DQ. Thus, the MHC Class II DR antigen is characteristic of relatively early stem cells.

Methods for isolation of HSCs and their maintenance and differentiation in culture are known in the art (Santiago-Schwartz *et al* 1992 J Leuk Biol 52:274-281; Charbord *et al* 1996 Br J Haematol 94: 449-454; Dao *et al* 1997 Blood 89: 446-456; Piacibello *et al* 1997 Blood 89: 2644-2653) and in WO91/09938. Methods for retroviral mediated transduction of HSCs and transfer to patients are also described (Dunbar *et al* 1996 Hum Gene Ther 7:231-253).

FUSION PROTEIN

In the present invention where a fusion protein is required, the domains are fused by constructing a hybrid DNA molecule. The hybrid molecule specifies the amino acid sequence of the additional molecule covalently linked to a DNA sequence that specifies all, or an active part, of the prodrug activation domain. If co-administration rather than fusion is required then two genes encoding the prodrug activation domain and the additional protein are incorporated into a suitable gene delivery vector (GDV) designed to ensure co-adminstration to a cell. For example, the additional protein can be expressed from a separate expression cassette in the same vector by using a distinct promoter / enhancer, alternatively the inclusion of two coding sequences separated by an internal ribosome entry site IRES will achieve co-administration.

Thus, in another aspect, the invention provides a nucleic acid encoding the at least one component of the agent described herein, said nucleic acid capable of expressing the at least one component of the agent in a target cell. Preferably, the invention provides a

nucleic acid encoding the fusion protein described herein, said nucleic acid capable of expressing the fusion protein in a target cell.

In another aspect the invention provides a nucleic acid vector containing the nucleic acid encoding the at least one component of the system, said vector capable of delivering the nucleic acid to a target cell. Preferably, the invention provides a nucleic acid vector containing the nucleic acid encoding the fusion protein, said vector capable of delivering the nucleic acid to a target cell. Suitable vectors according to the invention include viral vectors, in particular retroviral vectors.

NUCLEIC ACIDS

The invention also provides nucleic acid encoding the fusion proteins of the invention. These may be constructed using standard recombinant DNA methodologies. The nucleic acid may be RNA or DNA and is preferably DNA. Where it is RNA, manipulations may be performed via cDNA intermediates. Generally, a nucleic acid sequence encoding the first region will be prepared and suitable restriction sites provided at the 5' and/or 3' ends. Conveniently the sequence is manipulated in a standard laboratory vector, such as a plasmid vector based on pBR322 or pUC19 (see below). Reference may be made to Molecular Cloning by Sambrook *et al.* (Cold Spring Harbor, 1989) or similar standard reference books for exact details of the appropriate techniques.

Nucleic acid encoding the second region may likewise be provided in a similar vector system. Sources of nucleic acid may be ascertained by reference to published literature or databanks such as Genbank.

EXPRESSION VECTORS AND HOST CELLS

The nucleic acid encoding a fusion protein according to the invention, or constituent part(s) thereof, can be incorporated into vectors for further manipulation. As used

herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well within the skill of the artisan. Many vectors are available, and selection of appropriate vector will depend on the intended use of the vector, i.e. whether it is to be used for DNA amplification or for DNA expression, the size of the DNA to be inserted into the vector, and the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: an origin of replication, one or more marker genes, an enhancer element, a promoter, a transcription termination sequence and a signal sequence.

An expression vector includes any vector capable of expressing nucleic acids that are operatively linked with regulatory sequences, such as promoter regions, that are capable of expression of such DNAs. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector, that upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those with ordinary skill in the art and include those that are replicable in eukaryotic and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. For example, DNAs encoding the fusion protein according to the invention may be inserted into a vector suitable for expression of cDNAs in mammalian cells, e.g. a CMV enhancer-based vector such as pEVRF (Matthias, *et al.*, (1989) NAR 17, 6418).

Construction of vectors according to the invention employs conventional ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed plasmids is performed in a known fashion. Suitable methods for constructing expression vectors, preparing in vitro transcripts, introducing DNA into host cells, and performing analyses for assessing expression and function are known to those skilled in the art. Gene presence, amplification and/or

expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA analysis), or in situ hybridisation, using an appropriately labelled probe based on a sequence provided herein. Those skilled in the art will readily envisage how these methods may be modified, if desired.

DELIVERY SYSTEMS

In the present invention the hybrid DNA molecule may be delivered to the target cell population by any suitable Gene Delivery Vehicle, GDV. This includes but is not restricted to, DNA, formulated in lipid or protein complexes or administered as naked DNA via injection or biolistic delivery, viruses such as retroviruses, adenoviruses, herpes viruses, vaccinia viruses, adeno associated viruses. The GDV can be designed by a person ordinarily skilled in the art of recombinant DNA technology and gene expression to express the fusion protein at appropriate levels and with the cellular specificity demanded by a particular application. Alternatively the novel prodrug activating enzyme combinations are delivered by cells such as monocytes, macrophages, lymphocytes or hematopoietic stem cells. In particular a novel cell-dependent delivery system is used. In this system the genes encoding the prodrug activating proteins and protein combinations are introduced into a macrophage, monocyte or monocyte stem cell precursor *ex vivo* and then introduced into the patient.

As it is well known in the art, a vector is a tool that allows or faciliates the transfer of an entity from one environment to another. In accordance with the present invention, and by way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a target cell. Optionally, once within the target cell, the vector may then serve to maintain the heterologous DNA within the cell or may act as a unit of DNA replication. Examples of vectors used in recombinant DNA techniques include plasmids, chromosomes, artificial chromosomes or viruses.

The vector can be delivered by viral or non-viral techniques.

Non-viral delivery systems include but are not limted to DNA transfection methods. Here, transfection includes a process using a non-viral vector to deliver a gene to a target mammalian cell.

Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) (Nature Biotechnology 1996 14; 556), multivalent cations such as spermine, cationic lipids or polylysine, 1, 2,-bis (oleoyloxy)-3-(trimethylammonio) propane (DOTAP)-cholesterol complexes (Wolff and Trubetskoy 1998 Nature Biotechnology 16: 421) and combinations thereof.

Viral delivery systems include but are not limited to adenovirus vector, an adeno-associated viral (AAV) vector, a herpes viral vector, a retroviral vector, a lentiviral vector, a poxvirus, a pox-associated viral, a pox-lentiviral or a baculoviral vector.

Examples of retroviruses include but are not limited to: murine leukemia virus (MLV), human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV).

A detailed list of retroviruses may be found in Coffin *et al* ("Retroviruses" 1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763).

Details on the genomic structure of some retroviruses may be found in the art. By way f example, details on HIV and Mo-MLV may be found from the NCBI Genbank (Genome Accession Nos. AF033819 and AF033811, respectively).

Retroviruses may be broadly divided into two categories: namely, "simple" and "complex". Retroviruses may even be further divided into seven groups. Five of these groups represent retroviruses with oncogenic potential. The remaining two groups are the lentiviruses and the spumaviruses. A review of these retroviruses is presented in Coffin *et al.*, 1997 (*ibid*).

The lentivirus group can be split even further into "primate" and "non-primate". Examples of primate lentiviruses include human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

A distinction between the lentivirus family and other types of retroviruses is that lentiviruses have the capability to infect both dividing and non-dividing cells. In contrast, other retroviruses - such as MLV - are unable to infect non-dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue.

Each retroviral genome comprises genes called *gag*, *pol* and *env* which code for virion proteins and enzymes. In the provirus, these genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. LTRs also serve as enhancer-promoter sequences and can control the expression of the viral genes. Encapsidation of the retroviral RNAs occurs by virtue of a *psi* sequence located at the 5' end of the viral genome.

The LTRs themselves are identical sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA and U5 is derived from the sequence unique to the 5' end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

The basic molecular organisation of an infectious retroviral RNA genome is (5') R - U5 - *gag*, *pol*, *env* - U3-R (3'). In a defective retroviral vector genome *gag*, *pol* and *env* may be absent or not functional. The R regions at both ends of the RNA are repeated sequences. U5 and U3 represent unique sequences at the 5' and 3' ends of the RNA genome respectively.

Host range and tissue tropism varies between different retroviruses. In some cases, this specificity may restrict the transduction potential of a recombinant retroviral vector. For this reason, many gene therapy experiments have used MLV. A particular MLV that has an envelope protein called 4070A is known as an amphotropic virus, and this can also infect human cells because its envelope protein "docks" with a phosphate transport protein that is conserved between man and mouse. This transporter is ubiquitous and so these viruses are capable of infecting many cell types.

In some cases however, it may be beneficial, especially from a safety point of view, to target specifically restricted cells. Replacement of the *env* gene with a heterologous *env* gene is an example of a technique or strategy used to target specifically certain cell types. This technique is called pseudotyping and examples may be found in WO-A-98/05759, WO-A-98/05754, WO-A-97/17457, WO-A-96/09400 and WO-A-91/00047.

The term "recombinant retroviral vector" (RRV) refers to a vector with sufficient retroviral genetic information to allow packaging of an RNA genome, in the presence of packaging components, into a viral particle capable of infecting a target cell. Infection of the target cell includes reverse transcription and integration into the target cell

genome. The RRV in use typically carries non-viral coding sequences which are to be delivered by the vector to the target cell. An RRV is incapable of independent replication to produce infectious retroviral particles within the final target cell.

In a typical recombinant retroviral vector for use in gene therapy, at least part of one or more of the *gag, pol* and *env* protein coding regions essential for replication may be removed from the virus. This makes the retroviral vector replication-defective. The removed portions may even be replaced by an NOI to generate a virus capable of integrating its genome into a host genome but wherein the modified viral genome is unable to propagate itself due to a lack of structural proteins. When integrated in the host genome, expression of the NOI occurs - resulting in, for example, a therapeutic and/or a diagnostic effect. Thus, the transfer of an NOI into a site of interest is typically achieved by: integrating the NOI into the recombinant viral vector; packaging the modified viral vector into a virion coat; and allowing transduction of a site of interest - such as a targeted cell or a targeted cell population.

Replication-defective retroviral vectors are typically propagated, for example to prepare suitable titres of the retroviral vector for subsequent transduction, by using a combination of a packaging or helper cell line and the recombinant vector. That is to say, that the three packaging proteins can be provided *in trans*.

A "packaging cell line" contains one or more of the retroviral *gag, pol* and *env* genes. The packaging cell line produces the proteins required for packaging retroviral DNA but it cannot bring about encapsidation due to the lack of a *psi* region. However, when a recombinant vector carrying an NOI and a *psi* region is introduced into the packaging cell line, the helper proteins can package the *psi*-positive recombinant vector to produce the recombinant virus stock. This virus stock can be used to transduce cells to introduce the NOI into the genome of the target cells. It is preferred to use a *psi* packaging signal, called *psi* plus, that contains additional sequences spanning from upstream of the splice donor to downstream of the *gag* start codon (Bender *et al.*, 1987) since this has been shown to increase viral titres.

The recombinant virus whose genome lacks all genes required to make viral proteins can tranduce only once and cannot propagate. These viral vectors which are nly capable of a single round of transduction of target cells are known as replication defective vectors. Hence, the NOI is introduced into the host/target cell genome without the generation of potentially harmful retrovirus. A summary of the available packaging lines is presented in Coffin *et al.*, 1997 (*ibid*).

Retroviral packaging cell lines in which the *gag, pol* and *env* viral coding regions are carried on separate expression plasmids that are independently transfected into a packaging cell line are preferably used. This strategy, sometimes referred to as the three plasmid transfection method (Soneoka *et al.*, 1995), reduces the potential for production of a replication-competent virus since three recombinant events are required for wild type viral production. As recombination is greatly facilitated by homology, reducing or eliminating homology between the genomes of the vector and the helper can also be used to reduce the problem of replication-competent helper virus production.

An alternative to stably transfected packaging cell lines is to use transient transfected cell lines. Transient transfections may advantageously be used to measure levels of vector production when vectors are being developed. In this regard, transient transfection avoids the longer time required to generate stable vector-producing cell lines and may also be used if the vector or retroviral packaging components are toxic to cells. Components typically used to generate retroviral vectors include a plasmid encoding the gag/pol proteins, a plasmid encoding the env protein and a plasmid containing an NOI. Vector production involves transient transfection of one or more of these components into cells containing the other required components. If the vector encodes toxic genes or genes that interfere with the replication of the host cell, such as inhibitors of the cell cycle or genes that induce apotosis, it may be difficult to generate stable vector-producing cell lines, but transient transfection can be used to produce the vector before the cells die. Also, cell lines have been developed using transient transfection that produce vector titre levels that are comparable to the levels obtained

from stable vector-producing cell lines (Pear *et al.*, 1993).

It is highly desirable to use high-titre virus preparations in both experimental and practical applications. Techniques for increasing viral titre include using a *psi* plus packaging signal as discussed above and concentration of viral stocks. In addition, the use of different envelope proteins, such as the G protein from vesicular-stomatitis virus has improved titres following concentration to 10^9 per ml (Cosset *et al.*, 1995). Another technique is the split intron system for constructing retroviral vectors. This will be described later.

In addition to manipulating the retroviral vector with a view to increasing vector titre, retroviral vectors have also been designed to induce the production of a specific NOI in transduced cells. As already mentioned, the most common retroviral vector design involves the replacement of retroviral sequences with one or more NOIs to create replication-defective vectors. With regard to regulation of expression of the NOI, there are three main approaches currently in use.

1. The simplest approach has been to use the promoter in the retroviral 5' LTR to control the expresssion of a cDNA encoding an NOI or to alter the enhancer/promoter of the LTR to provide tissue-specific expression or inducibility. Where multiple NOIs are inserted, the additional downsteam NOIs can be expressed from a polycistronic mRNA by the use of internal ribosome entry sites.

2. The NOI may be operably linked to an internal heterologous promoter. This arrangement permits more flexibility in promoter selection. Additional NOIs can still be expressed from the 5'LTR or the LTR can be mutated to prevent expression following infection of a target cell.

3. The NOI is inserted together with regulatory control elements in the reverse orientation to the 5'LTR. Genomic sequences including enhancers, promoters, introns and 3' regions may be included. In this way it may be possible to achieve tightly

regulated tissue-specific gene expression.

In addition, we have now shown that there is a particular advantage to configuring retroviral vectors, in particular lentiviral vectors, as single transcription unit vectors whereby the HRE/promoter construct of the invention is placed within the 3'LTR such that the resultant duplication of the 3'LTR also leads to duplication of the regulatory sequence (i.e. the 5'LTR of the provirus will contain the HRE/promoter construct duplicated from the 3'LTR). We have now shown that this arrangement enhances the activated response to hypoxia in a synergistic manner. Consequently, it is preferred to use a retroviral vector which comprises an HRE/promoter of the invention within its LTR. More specifically, the HRE/promoter construct (optionally together with any additional regulatory sequences such as tissue-specific enhancer elements) may be inserted into the 3' U3 region of the retroviral vector or the 5' U5 region, most preferably the 3' U3 region. Preferably, the NOI is not also inserted into the LTR since the resulting two copies in the provirus can decrease the size of the NOI which can be accommodated by the retroviral vector. Instead, the NOI is preferably inserted into the region of the retroviral vector which is normally occupied by the *env* gene.

The NOI may or may not include a selectable marker. If the vector contains an NOI that is not a selectable marker, the vector can be introduced into packaging cells by co-transfection with a selectable marker present on a separate plasmid. This strategy has an appealing advantage for gene therapy in that a single protein is expressed in the ultimate target cells and possible toxicity or antigenicity of a selectable marker is avoided. However, when the inserted gene is not selectable, this approach has the disadvantage that it is more difficult to generate cells that produce a high titre vector stock. In addition it is usually more difficult to determine the titre of the vector.

The current methodologies used to design retroviral vectors that express two or more proteins have relied on three general strategies. These include: (i) the expression of different proteins from alternatively spliced mRNAs transcribed from one promoter; (ii) the use of the promoter in the 5' LTR and internal promoters to drive transcription f

different cDNAs and (iii) the use of internal ribosomal entry site (IRES) elements to allow translation of multiple coding regions from either a single mRNA or from fusion proteins that can then be expressed from an open reading frame.

Vectors containing internal heterologous promoters have been widely used to express multiple genes. An internal promoter makes it possible to exploit promoter/enhancer combinations other than the viral LTR for driving gene expression. Multiple internal promoters can be included in a retroviral vector and it has proved possible to express at least three different cDNAs each from its own promoter.

Lentiviruses

The lentivirus group can be into "primate" and "non-primate". Examples of primate lentiviruses include human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

A distinction between the lentivirus family and other types of retroviruses is that lentiviruses have the capability to infect both dividing and non-dividing cells. In contrast, other retroviruses - such as MLV - are unable to infect non-dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue. Thus, lentiviral vectors may advantageously be used in the present invention since lentiviruses are capable of infecting a wide range of non-dividing cells, by contrast to certain other retroviruses that require cell division for stable integration.

A number of vectors have been developed based on various members of the lentivirus sub-familiy of the retroviridae and a number of these are the subject of patent applications (WO-A-98/18815; WO-A-97/12622). Preferred lentiviral vectors are based

on HIV, SIV or EIAV. The simplest vectors constructed from HIV-1 have the complete HIV genome except for a deletion of part of the *env* coding region or replacement of the *nef* coding region. Notably these vectors express *gag/pol* and all of the accessory genes hence require only an envelope to produce infectious virus particles. Of the accessory genes *vif*, *vpr*, *vpu* and *nef* are non-essential. More recently however vectors have been described that are efficient yet lack most or all of the accessory factors, for example Blomer *et al.*, 1997 and Kim *et al.*, 1998. Thus a lentiviral vector of the invention preferably lacks at least one accessory gene, more preferably all accessory genes.

One preferred general format for HIV-based lentiviral vectors is, HIV 5'LTR and leader, some gag coding region sequences (to supply packaging functions), a reporter cassette, the rev response element (RRE) and the 3'LTR. In these vectors *gag/pol*, accessory gene products and envelope functions are supplied either from a single plasmid or from two or more co-transfected plasmids, or by co-infection of vector containing cells with HIV. More recently the lentiviral vector configurations have been further refined. For example self inactivating HIV vectors have been produced where the HIV LTR is deleted to restrict expression to the internal cassette (Myoshi *et al.*, 1998).

In a preferred embodiment, the lentiviral vector of the present invention is a non-primate lentiviral vector, for example EIAV. We have shown recently that the amount of vector genomic sequence required from a non-primate lentivirus to produce an efficient cloning vector is substantially less than has been described for an HIV vector. We have shown that a minimal EIAV vector lacking large regions of the gag gene suffices for efficient packaging and indeed leads to higher viral titres. Thus, a minimal EIAV genome vector typically comprises a promoter and optionally an enhancer capable of directing expression of a retroviral vector genome comprising, in order, the following elements from an EIAV: part of the 5'LTR containing an R-region and a U5 region; sequences from the 5'untranslated region of the gag gene containing a functional packaging signal and a portion of the gag coding sequence; an inserti n site for a gene of interest and a 3'LTR from EIAV. The 3' LTR may be a hybrid LTR containing at

least the polypurine tract and R-U5 region from an EIAV and sequences from another source to replace all or part of the U3 regi n. Alternatively, the R region in the 5' and 3' LTRs may be replaced. WO-A-96/33623 describes a method for replacing both the U3 and R regions of retroviral vector genomes.

Preferably, the portion of the gag gene contains less than the first 350 nucleotides of the gag coding region, more preferably only the first 150 or 109 nucleotides of the gag coding region, only about the first 109 nucleotides being especially preferred.

In a particularly preferred embodiment of the first aspect of the invention, a hCMV-MIE promoter enhancer is used to direct expression of the retroviral RNA transcript. The U3 region enhancer may, for example, be replaced by a hypoxia responsive enhancer (HRE) of the present invention and an SV40 or MLV promoter.

The minimal EIAV vector also lacks S2, Tat, Rev and dUTPase genes but may still be used in vector production or for transduction of dividing and non-dividing cells. The viral genome may typically be packaged in cells by providing gagpol and env functions in *trans*. For example DNA sequences encoding gagpol and env may be co-introduced into a cell along with the minimal vector as described above for retroviruses in general. Preferably the gagpol sequence is of non-primate lentiviral origin. It is particularly advantageous to include the leader sequences between the end of the 5' LTR and the ATG start codon of gag upstream of the gagpol coding sequence to provide for maximum gagpol expression. It may also be desirable to include the Rev and/or RRE sequences although this is not essential and may be eliminated by codon optimisation of the EIAV gagpol.

Retroviral Split Intron Technology

Transcription of the proviral DNA recreates the full length viral RNA genomic and subgenomic-sized RNA molecules that are generated by RNA processing. Typically, all RNA products serve as templates for the production of viral proteins. The

expression of the RNA products is achieved by a combination f RNA transcript splicing and ribosomal frameshifting during translation.

RNA transcript splicing is carried out in higher eukaryotic cells by nuclear RNA processing machinery (the spliceosome) that recognises short consensus sequences at the boundaries of the sequences to be spliced. These consensus splice sites follow the so-called 'GT-AG rule' (although in the RNA sequence GT is of course GU). The 5' site, or the splice donor (SD), has a highly conserved GT dinucleotide at the first exon-intron boundary and the 3' site, or the splice acceptor (SA), has a highly conserved AG dinucleotide at the second exon-intron boundary. These consensus sequences confer directionality on the splicing process. Splice sites have been shown to be generic - they do not have specificity for particular RNA precursors and can be spliced by any cell. The splicing process also depends on the presence of a sequence within the region to be spliced out called a branch site. The branch site is usually lies between 18 to 40 nucleotides upstream of the 3' SA and is believed to identify the nearest SA as the correct splice site for connection to the 5' SD.

Unlike most cellular mRNAs, in which all introns are efficiently spliced, newly synthesised retroviral RNA must be diverted into two populations. One population remains unspliced to serve as the genomic RNA and the other population is spliced to provide subgenomic RNA.

In simple retroviruses, one population of newly synthesised retroviral RNA remains unspliced to serve as the genomic RNA and as mRNA for *gag* and *pol*. The other population is spliced, fusing the 5' portion of the genomic RNA to the downstream genes, most commonly *env*. Splicing is achieved with the use of a splice donor positioned upstream of *gag* and a splice acceptor near the 3' terminus of *pol*. The intron between the splice donor and splice acceptor that is removed by splicing contains the *gag* and *pol* genes. This splicing event creates the mRNA for envelope (Env) protein. Typically the splice donor is upstream of *gag* but in some viruses, such as ASLV, the splice donor is positioned a few codons into the *gag* gene resulting in a primary Env

translation product that includes a few amino-terminal amino acid residues in common with Gag. The Env protein is synthesised on membrane-bound polyribosomes and transported by the cell's vesicular traffic to the plasma membrane, where it is incorporated into viral particles.

Complex retroviruses generate both singly and multiply spliced transcripts that encode not only the *env* gene products but also the sets of regulatory and accessory proteins unique to these viruses. Compex retroviruses such as the lentiviruses, and especially HIV, provide striking examples of the complexity of alternative splicing that can occur during retroviral infection. For example, it is now known that HIV-1 is capable of producing over 30 different mRNAs by sub-optimal splicing from primary genomic transcripts. This selection process appears to be regulated as mutations that disrupt competing splice acceptors can cause shifts in the splicing patterns and can affect viral infectivity.

The ability of higher eukaryotic cells to splice sequences between a 5' SD and a 3' SA can be utilised by retroviral vectors according to the present invention. In split-intron technology, a retroviral vector is constructed which comprises a first nucleotide sequence (NS) capable of yielding a functional splice donor site (FSDS) and a second NS capable of yielding a functional splice acceptor site (FSAS); wherein the first NS is downstream of the second NS and is present in the sequences within the 3' LTR (the U3 region) of the retroviral vector that are duplicated in the 5' LTR as a result of reverse transcription of the RNA form of the retroviral vector into the DNA form that integrates into a host cell genome. Since the first NS capable of yielding an FSDS is downstream of the second NS capable of yielding an FSAS, splicing will not occur in the retroviral vector using these sites. However, in the reverse-transcribed DNA form of the vector, such as the integrated provirus, the first NS is now upstream of the second NS and thus a functional 5' splice donor - 3' splice acceptor pairing is generated. Consequently, when transcription occurs from the provirus, the resulting RNA is capable of being spliced to remove intervening sequences between the FSDS and the FSAS. Depending

on the nature of the sequences which are spliced from RNA transcripts produced from the provirus, this process has several applicati ns.

A preferred embodiment in which split-intron technology may preferably be used relates to a hybrid vector system (see above). In this system, the retroviral genome delivered by the primary viral vector, such as an adenoviral vector, to a primary target celll is in a configuration such the the first NS is downstream of the second NS. Thus, splicing out of sequences between the first NS and second NS using the first NS as a splice donor and the second NS as a splice acceptor should not occur since they are in the wrong orientation. However, the viral particles packaged by the primary target cell may be used to infect a secondary target cell. In the secondary target cell, reverse transcription and integration of the viral genome will typically occur. This will result in a functional splice donor/splice acceptor configuration since the FSDS will then be located in the 5' LTR, i.e. upstream of the FSAS. Splicing may then occur to remove the sequences between the FSDS and FSAS.

The nucleotide sequence between the 5' LTR and the second NS in the retroviral vector that is found between the FSAS and FSDS in the provirus may contain a nucleic acid sequence of interest (NOI). The NOI may contain sequences that are essential for production of viral particles, for example env or gagpol sequences. Since the NOI will be spliced out from proviral transcripts, the provirus will not be able to give rise to viable viral particles. This will, for example, allow construction of adeno retroviral vectors that contain NOIs encoding viral components and gene products of interest, such as therapeutic polypeptides, that have the added safety feature of not being able to produce said viral components when the viral particles assembled in the primary target cells are introduced into secondary target cells.

A further embodiment allows the expression of an NOI in a secondary target cell and not a primary target cell. In one alternative of this embodiment, removal of the intervening sequences between the FSDS and the FSAS in the proviral RNA transcript brings a regulatory sequence that is 5' to the FSDS in the proviral genome near to the 5'

end of an NOI that was 3' to the FSAS in the proviral genome such that the regulatory sequence is now operably linked to the NOI and can direct transcription from the NOI whereas when the intervening sequences were present, the regulatory sequence was not operably linked to the NOI. Although the regulatory sequence may be upstream of the NOI in the retroviral vector, in a preferred embodiment the regulatory sequence is upstream of the first NS in the retroviral vector, within the U3 region of the 3' LTR such that it is downstream of the NOI. Thus, only when reverse transcription of the retroviral RNA takes place in the secondary target cells is the regulatory sequence positioned 5' of the NOI.

In summary, therefore, NOIs which are positioned between the FSDS and the FSAS (and are therefore upstream of the second NS in the retroviral vector) are sequences that will typically be spliced out of the viral RNA transcript produced by the provirus in the secondary target cell. Such NOIs may therefore include, but are not limited to, retroviral sequences required for assembly of retroviral particles in the primary cells. These include env sequences, gagpol sequences, sequences encoding essential accessory genes and/or packaging sequences. NOIs which are positioned downstream of the FSAS in the proviral genome (and therefore also downstream of the second NS in the retroviral vector) will therefore typically include, but are generally not limited to, sequences encoding gene products that it is desired to express in the secondary target cells, such as therapeutic products.

It will be appreciated that in the context of an adenoretroviral hybrid system, the reference to retroviral vector includes the retroviral genome sequences present in a primary adenoretroviral vector as well as the subsequent RNA genome produced in the primary target cells and subsequently packaged into viral particles.

As discussed above, functional splicing sequences also require a branch sequence which is found 5' and near to the FSAS (and consequently to the second NS) and therefore a suitable branch sequence should be present. It should also be appreciated that construction of split-intron vectors may required functional inactivation of existing

functional splice donor sites, for example to prevent splicing in the retroviral RNA genome transcribed in the primary target cells. Typically, this may be achieved by mutating the GT dinucleotide consensus, for example to GC, using standard techniques.

Adenoviruses

The adenovirus is a double-stranded, linear DNA virus that does not go through an RNA intermediate. There are over 50 different human serotypes of adenovirus divided into 6 subgroups based on the genetic sequence homology all of which exhibit comparable genetic organisation. Human adenovirus group C serotypes 2 and 5 (with 95% sequence homology) are most commonly used in adenoviral vector systems and are normally associated with upper respiratory tract infections in the young.

The adenoviruses/adenoviral vectors of the invention may be of human or animal origin. As regards the adenoviruses of human origin, preferred adenoviruses are those classified in group C, in particular the adenoviruses of type 2 (Ad2), 5 (Ad5), 7 (Ad7) or 12 (Ad12). More preferably, it is an Ad2 or Ad5 adenovirus. Among the various adenoviruses of animal origin, canine adenovirus, mouse adenovirus or an avian adenovirus such as CELO virus (Cotton *et al.*, 1993) may be used. With respect to animal adenoviruses it is preferred to use adenoviruses of canine origin, and especially the strains of the CAV2 adenoviruses [manhattan strain or A26/61 (ATCC VR-800) for example]. Other adenoviruses of animal origin include those cited in application WO-A-94/26914 incorporated herein by reference.

As mentioned above, the organisation of the adenovirus genome is similiar in all of the adenovirus groups and specific functions are generally positioned at identical locations for each serotype studied. The genome of adenoviruses comprises an inverted terminal repeat (ITR) at each end, an encapsidation sequence (Psi), early genes and late genes. The main early genes have been classified into an array of intermediate early (E1a), delayed early (E1b, E2a, E2b, E3 and E4), and intermediate regions (see diagram below). Among these, the genes contained in the E1 region in particular are necessary

for viral propagation. The main late genes are contained in the L1 to L5 regions. The genome of the Ad5 adenovirus has been completely sequenced and is available on a database (see particularly Genbank Accession No. M73260). Likewise, parts, or even all of other adenoviral genomes (such as Ad2, Ad7, Ad12) have also been sequenced.

For use as recombinant vectors, an adenovirus is typically modified so as to make it incapable of replicating in an infected cell.

Thus, constructs described in the prior art include adenoviruses deleted for the E1 region, essential for viral replication, into which are inserted the heterologous DNA sequences (Levrero *et al.*, 1991; Gosh-Choudhury *et al.*, 1986). Moreover, to improve the properties of the vector, it has been proposed to create other deletions or modifications in the adenovirus genome. Thus, a heat-sensitive point mutation has been introduced into the ts125 mutant, making it possible to inactivate the 72 kDa DNA-binding protein (DBP). Preferably, a recombinant adenoviral vector used in the invention comprises a deletion in the E1 region of its genome. More particularly, it comprises a deletion in the E1a and E1b regions. According to a particularly preferred mode, the E1 region is inactivated by deletion of a *PvuII-BglII* fragment stretching from nucleotide 454 to nucleotide 3328, in the Ad5 adenovirus sequence (Genbank Accession No. M73260). In another preferred embodiment, the E1 region is inactivated by deletion of an *HinfII-Sau3A* fragment stretching from nucleotide 382 to nucleotide 3446.

Other adenoviral vectors comprise a deletion of another region essential for viral replication and/or propagation, the E4 region. The E4 region is involved in the regulation of the expression of the late genes, in the stability of the late nuclear RNAs, in decreasing host cell protein expression and in the efficiency of the replication of the viral DNA. Adenoviral vectors in which the E1 and E4 regions are deleted therefore possess very reduced viral gene expression and transcriptional background noise. Such vectors have been described for example in applications WO-A-94/28152, WO-A-95/02697, WO-A-96/22378. In addition, vectors carrying a modification of the IVa2 gene have also been described (WO-A-96/10088).

According to a preferred variant, a recombinant adenoviral vector used in the invention comprises, in addition, a deletion in the E4 region of its genome. More particularly, the deletion in the E4 region affects all the open reading frames. There may be mentioned, by way of a precise example, deletions of nucleotides 33466-35535 or 33093-35535. In particular, preferred vectors comprise a deletion of the whole of the E4 region. This may be carried deletion or excision of an *MaeII-MscI* fragment corresponding to nucleotides 35835-32720. Other types of deletions in the E4 region are described in applications WO-A-95/02697 and WO-A-96/22378, incorporated herein by reference.

Alternatively, only a functional part of E4 is deleted. This part comprises at least the ORF3 and ORF6 frames. By way of example, these coding frames can be deleted from the genome in the form of *PvuII-AluI* and *BglII-PvuII* fragments respectively, corresponding to nucleotides 34801-34329 and 34115-33126 respectively. The deletions of the E4 region of the virus Ad2 dl808 or of viruses Ad5 dl1004, Ad5 dl1007, Ad5 dl1011 or Ad5 dl1014 can also be used within the framework of the invention.

The positions given above refer to the wild-type Ad5 adenovirus sequence as published and accessible on a database. Although minor variations may exist between the various adenovirus serotypes, these positions are generally applicable to the construction of recombinant adenoviruses according to the invention from any serotype, and especially the adenoviruses Ad2 and Ad7.

Moreover, the adenoviruses produced may possess other alterations in their genome. In particular, other regions may be deleted to increase the capacity of the virus and reduce its side effects linked to the expression of viral genes. Thus, all or part of the E3 or IVa2 region in particular may be deleted. As regards the E3 region, it may however be particularly preferred to conserve the part encoding the gp19K protein. This protein indeed makes it possible to prevent the adenoviral vector from becoming the subject of an immune reaction which (i) would limit its action and (ii) could have undesirable side effects. According to a specific mode, the E3 region is deleted and the sequence

encoding the gp19K protein is reintroduced under the control f a heterologous promoter.

The polynucleotide of the invention/NOI can be inserted into various sites of the recombinant genome. It can be inserted at into the E1, E3 or E4 region, as a replacement for the deleted or surplus sequences. It can also be inserted into any other site, outside the sequences necessary *in cis* for the production of the viruses (ITR sequences and encapsidation sequence).

The E2 region is essential as it encodes the 72 kDa DNA binding protein, DNA polymerase and the 80 kDa precurser of the 55 kDa Terminal Protein (TP) needed for protein priming to initiate DNA synthesis.

An alternative approach to making a more defective virus has been to "gut" the virus completely maintaining only the terminal repeats required for viral replication. The "gutted" or "gutless" viruses can be grown to high titres with a first generation helper virus in the 293 cell line.

The recombinant adenoviruses are typically produced in an encapsidation cell line, which is a cell line capable of complementing *in trans* one or more of the functions deficient in the recombinant adenoviral genome. One of these lines is for example line 293, into which part of the adenovirus genome has been integrated. More precisely, line 293 is a human kidney embryonic cell line containing the left end (about 11-12%) of the genome of serotype 5 adenovirus (Ad5), comprising the left ITR, the encapsidation region, the E1 region, including E1a and E1b, the region encoding protein pIX and part of the region encoding protein pIVa2. This line is capable of transcomplementing recombinant adenoviruses defective for the E1 region, that is to say lacking all or part of the E1 region, and of producing viral stocks having high titres. This line is also capable of producing, at a permissive temperature (32°C), virus stocks comprising, in addition, the heat-sensitive E2 mutation.

Other cell lines capable of complementing the E1 region have been described, based in particular n human lung carcinoma cells A549 (WO-A-94/28152) or on human retinoblasts (Hum. Gen. Ther. (1996) 215). Moreover, cell lines capable of transcomplementing several adenovirus functions have also been described, for example cell lines complementing the E1 and E4 regions (Yeh *et al.*, 1996a, b; Krougliak *et al.*, 1995) and lines complementing the E1 and E2 regions (WO-A-94/28152, WO-A-95/02697, WO-A-95/27071).

The recombinant adenoviruses are usually produced by introducing the viral DNA into the encapsidation line, followed by lysis of the cells after about 2 or 3 days (the kinetics of the adenoviral cycle being 24 to 36 hours). For carrying out the process, the viral DNA introduced may be the complete recombinant viral genome, optionally constructed in a bacterium (WO-A-96/25506) or in a yeast (WO-A-95/03400), transfected into the cells. It may also be a recombinant virus used to infect the encapsidation line. The viral DNA may also be introduced in the form of fragments each carrying part of the recombinant viral genome and a region of homology which makes it possible, after introduction into the encapsidation cell, to reconstitute the recombinant viral genome by homologous recombination between the various fragments.

Replication-competent adenoviruses can also be used for gene therapy. For example, the E1a gene can be inserted into a first generation virus under the regulation of a tumour-specific promoter. In theory, following injection of the virus into a tumour, it could replicate specifically in the tumour but not in the surrounding normal cells. This type of vector could be used either to kill tumour cells directly by lysis or to deliver a "suicide gene" such as the herpes-simplex-virus thymidine-kinase gene (HSV *tk*) which can kill infected and bystander cells following treatment with ganciclovir.

The viral genes may be placed under the control of a hypoxia responsive regulatory element.

Hybrid vector systems

Hybrid adenovirus/retrovirus systems may also be used whereby the features of adenoviruses are combined with the genetic stability of retroviruses. These hybrid viral vectors use the adenovirus to transduce target cells when then become transient retroviral producer cells that can stably infect neighbouring cells.

A hybrid viral vector system of the present invention comprises one or more primary viral vectors which encode a secondary viral vector, the primary vector or vectors being capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell.

Thus a genetic vector of the invention consists of a primary vector manufactured *in vitro* which encodes the genes necessary to produce a secondary vector *in vivo*. In use, the secondary vector carries one or more selected genes for insertion into the secondary target cell. The selected genes may be one or more marker genes and/or therapeutic genes (see above).

The primary viral vector or vectors may be a variety of different viral vectors, such as retroviral (including lentiviral), adenoviral, herpes virus or pox virus vectors, or in the case of multiple primary viral vectors, they may be a mixture of vectors of different viral origin. In whichever case, the primary viral vectors are preferably defective in that they are incapable of independent replication. Thus, they are capable of entering a target cell and delivering the secondary vector sequences, but not of replicating so as to go on to infect further target cells.

In the case where the hybrid viral vector system comprises more than one primary vector to encode the secondary vector, all of the primary vectors will be used to infect a primary target cell population, usually simultaneously. Preferably, there is a single primary viral vector which encodes all components of the secondary viral vector.

The preferred single or multiple primary viral vectors are adenoviral vectors. Adenovirus vectors have significant advantages over other viral vectors in terms of the titres which can be obtained from *in vitro* cultures. The adenoviral particles are also comparatively stable compared with those of enveloped viruses and are therefore more readily purified and stored.

The secondary viral vector is preferably a retroviral vector, more preferably a lentiviral vector. The secondary vector is produced by expression of essential genes for assembly and packaging of a defective viral vector particle, within the primary target cells. It is defective in that it is incapable of independent replication. Thus, once the secondary retroviral vector has transduced a secondary target cell, it is incapable of spreading by replication to any further target cells.

The secondary vector may be produced from expression of essential genes for retroviral vector production encoded in the DNA of the primary vector. Such genes may include a gag-pol gene from a retrovirus, an envelope gene from an enveloped virus and a defective retroviral genome containing one or more therapeutic genes. The defective retroviral genome contains in general terms sequences to enable reverse transcription, at least part of a 5' long terminal repeat (LTR), at least part of a 3'LTR and a packaging signal.

Importantly, the secondary vector is also safe for *in vivo* use in that incorporated into it are one or more safety features which eliminate the possibility of recombination to produce an infectious virus capable of independent replication.

To ensure that it is replication defective the secondary vector may be encoded by a plurality of transcription units, which may be located in a single or in two or more adenoviral or other primary vectors. Thus, there may be a transcription unit encoding the secondary vector genome, a transcription unit encoding *gag-pol* and a transcription unit encoding *env*. Alternatively, two or more of these may be combined. For example,

nucleic acid sequences encoding *gag-pol* and *env*, or *env* and the genome, may be combined in a single transcription unit. Ways of achieving this are known in the art.

Transcription units as described herein are regions of nucleic acid containing coding sequences and the signals for achieving expression of those coding sequences independently of any other coding sequences. Thus, each transcription unit generally comprises at least a promoter, an enhancer and a polyadenylation signal. The promoter and enhancer of the transcription units encoding the secondary vector are preferably strongly active, or capable of being strongly induced, in the primary target cells under conditions for production of the secondary viral vector. The promoter and/or enhancer may be constitutively efficient, or may be tissue or temporally restricted in their activity.

Safety features which may be incorporated into the hybrid viral vector system are described below. One or more such features may be present.

Firstly, sequence homology between the sequences encoding the components of the secondary vector may be avoided by deletion of regions of homology. Regions of homology allow genetic recombination to occur. In a particular embodiment, three transcription units are used to construct a secondary retroviral vector. A first transcription unit contains a retroviral *gag-pol* gene under the control of a non-retroviral promoter and enhancer. A second transcription unit contains a retroviral *env* gene under the control of a non-retroviral promoter and enhancer. A third transcription unit comprises a defective retroviral genome under the control of a non-retroviral promoter and enhancer. In the native retroviral genome, the packaging signal is located such that part of the *gag* sequence is required for proper functioning. Normally when retroviral vector systems are constructed therefore, the packaging signal, including part of the *gag* gene, remains in the vector genome. In the present case however, the defective retroviral genome contains a minimal packaging signal which does not contain sequences homologous to *gag* sequences in the first transcription unit. Also, in retroviruses, for example Moloney Murine Leukaemia virus (MMLV), there is a small

region of overlap between the 3' end of the *pol* coding sequence and the 5' end of *env*. The corresponding region of homology between the first and second transcription units may be removed by altering the sequence of either the 3' end of the *pol* coding sequence or the 5' end of *env* so as to change the codon usage but not the amino acid sequence of the encoded proteins.

Secondly, the possibility of replication competent secondary viral vectors may be avoided by pseudotyping the genome of one retrovirus with the envelope protein of another retrovirus or another enveloped virus so that regions of homology between the *env* and *gag-pol* components are avoided. In a particular embodiment the retroviral vector is constructed from the following three components. The first transcription unit contains a retroviral *gag-pol* gene under the control of a non-retroviral promoter and enhancer. The second transcription unit contains the *env* gene from the alternative enveloped virus, under the control of a non-retroviral promoter and enhancer. The third transcription unit comprises a defective retroviral genome under the control of a non-retroviral promoter and enhancer. The defective retroviral genome contains a minimal packaging signal which does not contain sequences homologous to *gag* sequences in the first transcription unit.

Pseudotyping may involve for example a retroviral genome based on a lentivirus such as an HIV or equine infectious anaemia virus (EIAV) and the envelope protein may for example be the amphotropic envelope protein designated 4070A. Alternatively, the retroviral genome may be based on MMLV and the envelope protein may be a protein from another virus which can be produced in non-toxic amounts within the primary target cell such as an Influenza haemagglutinin or vesicular stomatitis virus G protein. In another alternative, the envelope protein may be a modified envelope protein such as a mutant or engineered envelope protein. Modifications may be made or selected to introduce targeting ability or to reduce toxicity or for another purpose.

Thirdly, the possibility of replication competent retroviruses can be eliminated by using two transcription units constructed in a particular way. The first transcription unit

contains a *gag-pol* coding region under the control of a promoter-enhancer active in the primary target cell such as a hCMV promoter-enhancer or a tissue restricted promoter-enhancer. The second transcription unit encodes a retroviral genome RNA capable of being packaged into a retroviral particle. The second transcription unit contains retroviral sequences necessary for packaging, integration and reverse transcription and also contains sequences coding for an *env* protein of an enveloped virus and the coding sequence of one or more therapeutic genes.

In a preferred embodiment the hybrid viral vector system according to the invention comprises single or multiple adenoviral primary vectors which encodes or encode a retroviral secondary vector. Adenoviral vectors for use in the invention may be derived from a human adenovirus or an adenovirus which does not normally infect humans. Preferably the vectors are derived from Adenovirus Type 2 or adenovirus Type 5 (Ad2 or Ad5) or a mouse adenovirus or an avian adenovirus such as CELO virus. The vectors may be replication competent adenoviral vectors but are more preferably defective adenoviral vectors. Adenoviral vectors may be rendered defective by deletion of one or more components necessary for replication of the virus. Typically, each adenoviral vector contains at least a deletion in the E1 region. For production of infectious adenoviral vector particles, this deletion may be complemented by passage of the virus in a human embryo fibroblast cell line such as human 293 cell line, containing an integrated copy of the left portion of Ad5, including the E1 gene. The capacity for insertion of heterologous DNA into such vectors can be up to approximately 7 kb. Thus such vectors are useful for construction of a system according to the invention comprising three separate recombinant vectors each containing one of the essential transcription units for construction of the retroviral secondary vector.

Alternative adenoviral vectors are known in the art which contain further deletions in other adenoviral genes and these vectors are also suitable for use in the invention. Several of these second generation adenoviral vectors show reduced immunogenicity (eg E1 + E2 deletions Gorziglia *et al.*, 1996; E1 + E4 deleti ns Yeh *et al.*, 1996). Extended deletions serve to provide additional cloning capacity for the introduction of

multiple genes in the vector. For example a 25 kb deletion has been described (Lieber *et al.*, 1996) and a cl ning vector deleted of all viral genes has been reported (Fisher *et al.*, 1996) which will permit the introduction of more than 35 kb of heterologous DNA. Such vectors may be used to generate an adenoviral primary vector according to the invention encoding two or three transcription units for construction of the retroviral secondary vector.

Embodiments of the invention described solve one of the major problems associated with adenoviral and other viral vectors, namely that gene expression from such vectors is transient. The retroviral particles generated from the primary target cells can infect secondary target cells and gene expression in the secondary target cells is stably maintained because of the integration of the retroviral vector genome into the host cell genome. The secondary target cells do not express significant amounts of viral protein antigens and so are less immunogenic than the cells transduced with adenoviral vector.

The use of a retroviral vector as the secondary vector is also advantageous because it allows a degree of cellular discrimination, for instance by permitting the targeting of rapidly dividing cells. Furthermore, retroviral integration permits the stable expression of therapeutic genes in the target tissue, including stable expression in proliferating target cells.

Preferably, the primary viral vector preferentially infects a certain cell type or cell types. More preferably, the primary vector is a targeted vector, that is it has a tissue tropism which is altered compared to the native virus, so that the vector is targeted to particular cells. The term "targeted vector" is not necessarily linked to the term "target cell". "Target cell" simply refers to a cell which a vector, whether native or targeted, is capable of infecting or transducing.

Primary target cells for the vector system according to the invention include but are not limited to haematopoietic cells (including monocytes, macrophages, lymphocytes,

granulocytes or progenitor cells of any of these); endothelial cells; tumour cells; stromal cells; astrocytes or glial cells; muscle cells; and epithelial cells.

Thus, a primary target cell according to the invention, capable of producing the second viral vector, may be of any of the above cell types. In a preferred embodiment, the primary target cell according to the invention is a monocyte or macrophage infected by a defective adenoviral vector containing a first transcription unit for a retroviral gag-pol and a second transcription unit capable of producing a packageable defective retroviral genome. In this case at least the second transcription unit is preferably under the control of a promoter-enhancer which is preferentially active in a diseased location within the body such as an ischaemic site or the micro-environment of a solid tumour. In a particularly preferred embodiment of this aspect of the invention, the second transcription unit is constructed such that on insertion of the genome into the secondary target cell, an intron is generated which serves to reduce expression of the viral *env* gene and permit efficient expression of a therapeutic gene.

The secondary viral vectors may also be targeted vectors. For retroviral vectors, this may be achieved by modifying the envelope protein. The envelope protein of the retroviral secondary vector needs to be a non-toxic envelope or an envelope which may be produced in non-toxic amounts within the primary target cell, such as for example a MMLV amphotropic envelope or a modified amphotropic envelope. The safety feature in such a case is preferably the deletion of regions or sequence homology between retroviral components.

The secondary target cell population may be the same as the primary target cell population. For example delivery of a primary vector of the invention to tumour cells leads to replication and generation of further vector particles which can transduce further tumour cells. Alternatively, the secondary target cell population may be different from the primary target cell population. In this case the primary target cells serve as an endogenous factory within the body f the treated individual and produce additional vector particles which can infect the secondary target cell population. F r

example, the primary target cell population may be haematopoietic cells transduced by the primary vector *in vivo* or *ex vivo*. The primary target cells are then delivered to r migrate to a site within the body such as a tumour and produce the secondary vector particles, which are capable of transducing for example tumour cells within a solid tumour.

The invention permits the localised production of high titres of defective retroviral vector particles *in vivo* at or near the site at which action of a therapeutic protein or proteins is required with consequent efficient transduction of secondary target cells. This is more efficient than using either a defective adenoviral vector or a defective retroviral vector alone.

The invention also permits the production of retroviral vectors such as MMLV-based vectors in non-dividing and slowly-dividing cells *in vivo*. It had previously been possible to produce MMLV-based retroviral vectors only in rapidly dividing cells such as tissue culture-adapted cells proliferating *in vitro* or rapidly dividing tumour cells *in vivo*. Extending the range of cell types capable of producing retroviral vectors is advantageous for delivery of genes to the cells of solid tumours, many of which are dividing slowly, and for the use of non-dividing cells such as endothelial cells and cells of various haematopoietic lineages as endogenous factories for the production of therapeutic protein products.

PROMOTERS/ENHANCERS

By inducible or regulated we mean that expression may be preferabltially activated, e.g. expression may be altered in response to extracellular cues. By constitutive expression we mean produced in substantially constant amount; opposite of regulated. Constitutive expression, e.g. occurs continuously without requiring an external stimulus.

Preferably, the nucleic acid in a vector according to the invention is operatively linked to an expression control sequence capable of causing preferential expression of the

fusi n protein in the target cell. The expression control sequence may be for example a prom tor or enhancer which is preferentially active in certain cell types including the target cell, or a promotor or enhancer which is preferentially active under certain conditions such as hypoxic conditions. Expression control sequences which are capable of causing preferential expression under conditions of hypoxia are known as hypoxia responsive elements (Dachs *et al* 1997 Nature Medicine 3, 515).

The nucleic acid may be placed under the control of a promoter that is specifically activated, for example by hypoxia, only when the macrophage has entered the tumour. This facilitates local delivery of prodrug activating enzymes. This aspect of the invention is particularly useful for those prodrugs, for example Tirapazamine, RSU1069, EO9 and Mitomycin C, that are themselves markedly activated in the conditions such as hypoxia that exist preferentially in tumours.

The nucleic acid may be under the expression control of an expression regulatory element, usually a promoter or a promoter and enhancer. The enhancer and/or promoter may be preferentially active in a hypoxic or ischaemic or low glucose environment, such that the nucleic acid is preferentially expressed in the particular tissues of interest, such as in the environment of a tumour, arthritic joint or other sites of ischaemia. Thus any significant biological effect or deleterious effect of the nucleic acid on the individual being treated may be reduced or eliminated. The enhancer element or other elements conferring regulated expression may be present in multiple copies. Likewise, or in addition, the enhancer and/or promoter may be preferentially active in one or more specific cell types - such as any one or more of macrophages, endothelial cells or combinations thereof. Further examples include include respiratory airway epithelial cells, hepatocytes, muscle cells, cardiac myocytes, synoviocytes, primary mammary epithelial cess and post-mitotically terminally differentiated non-replicating cells such as macrophages neurons.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression.

The term "enhancer" includes a DNA sequence which binds to other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.

Preferably the promoters of the present invention are tissue specific. That is, they are capable of driving transcription of a nucleic acid in one tissue while remaining largely "silent" in other tissue types.

The term "tissue specific" means a promoter which is not restricted in activity to a single tissue type but which nevertheless shows selectivity in that they may be active in one group of tissues and less active or silent in another group. A desirable characteristic of the promoters of the present invention is that they posess a relatively low activity in the absence of activated hypoxia-regulated enhancer elements, even in the target tissue. One means of achieving this is to use "silencer" elements which suppress the activity of a selected promoter in the absence of hypoxia.

A number of tissue specific promoters, described above, may be particularly advantageous in practising the present invention. In most instances, these promoters may be isolated as convenient restriction digestion fragments suitable for cloning in a selected vector. Alternatively, promoter fragments may be isolated using the polymerase chain reaction. Cloning of the amplified fragments may be facilitated by incorporating restriction sites at the 5' end of the primers.

Promoters suitable for cardiac-specific expression include the promoter from the murine cardiac α-myosin heavy chain (MHC) gene. Suitable vascular endothelium-specific promoters include the Et-1 promoter and von Willebrand factor promoter.

Prostate specific promoters include the 5'flanking resion of the human glandular kallikrein-1 (hKLK2) gene and the prostate specific antigen (hKLK3).

Examples of promoters/enhancers which are cell specific include a macrophage-specific promoter or enhancer, such as CSF-1 promoter-enhancer, or elements from a mannose receptor gene promoter-enhancer (Rouleux *et al* 1994 Exp Cell Res 214:113-119). Alternatively, promoter or enhancer elements which are preferentially active in neutrophils, or a lymphocyte-specific enhancer such as an IL-2 gene enhancer, may be used.

The elevated expression of a therapeutic gene under hypoxic conditions can be induced by the presence of one or more hypoxic response enhancer (HRE) elements. HRE elements contain polynucleotide sequences that may be located either upstream (5') or downstream (3') of the promoter and/or therapeutic gene. The HRE enhancer element (HREE) is typically a *cis*-acting element, usually about 10-300 bp in length, that acts on a promoter to increase the transcription of a gene under the control of the promoter. Preferably, the promoter and enhancer elements are selected such that expression of a gene regulated by those elements is minimal in the presence of a healthy supply of oxygen and is upregulated under hypoxic or anoxic conditions.

The term "hypoxia" means a condition under which a particular organ or tissue receives an inadequate supply of oxygen.

The hypoxia response element may also be selected from, for example, the erythropoietin HRE element (HREE1), muscle pyruvate kinase (PKM), HRE element, B-enolase (enolase 3; ENO3) HRE element, endothelin-1 (ET-1)HRE element and metallothionein II (MTII) HRE element.

A further example of a hypoxia regulated enhancer is a binding element for the transcription factor HIF-1 (Dachs *et al* 1997 Nature Med 5: 515; Wang and Sememnza 1993 Proc Natl Acad Sci USA 90:4304; Firth *et al* 1994 Proc Natl Acad Sci USA 91: 6496). Hypoxia response enhancer elements have also been found in association with a number of genes including the erythropoietin (EPO) gene (Madan *et al* 1993 Proc Natl

Acad Sci 90: 3928; Semenza and Wang 1992 Mol Cell Biol 1992 12: 5447-5454). Other HREEs have been isolated from regulatory regions of both the muscle glycolytic enzyme pyrivate kinase (PKM) gene (Takenaka *et al* 1989 J Biol Chem 264: 2363-2367), the human muscle-specific β-enolase gene (ENO3; Peshavaria and Day 1991 Biochem J 275: 427-433) and the endothelin-1 (ET-1) gene (Inoue *et al* 1989 J Biol Chem 264: 14954-14959).

When the enzyme activation domain is expressed from a macrophage the promoter and/or enhancer should be constitutively efficient. Examples of constitutive promoters such as cytomegalovirus (CMV) are known in the art and any may be used. One preferred promoter-enhancer combination is a human cytomegalovirus (hCMV) major immediate early (MIE) promoter/enhancer combination. We have found that macrophages engineered to express prodrug activating enzymes such a P450 constitutively give particularly good results, for example in the reatment of cancer. This is particularly surprising.

HOST CELLS AND TARGET CELLS

Polynucleic acid constructs, nucleic acid vectors and viral vectors of the invention may be introduced into a variety of host cells. Host cells include both prokaryotic, for example bacterial, and eukaryotic, for example yeast and higher eukaryotic cells (such as insect, mammalian, for example human, cells). Host cells may be used to propagate both non-viral and viral vectors, for example to prepare nucleic acid vectors comprising a polynucleotide of the invention or to prepare high titre viral stocks.

Alternatively, host cells comprising a polynucleic acid sequence of the invention/NOI may be used in therapy, such as the use of macrophages discussed below in, for example, *ex vivo* therapy.

Non-viral nucleic acids and viral vectors are typically introduced into host cells using

techniques well known in the art such as transformation or transfection. Viral vectors may also be introduced into host cells using by infection.

Target cells, in the context of the present invention, means cells of, or from, an organism that typically it is desired to treat, rather than simply cell lines. Target cells may be removed from the organism and subsequently returned after treatment, or targeted *in vivo*. Thus for example, tumour cells *in vivo* can be considered to be target cells.

Examples of target cells include tumour cells (see below for list of tumours), in particular, tumour cells under conditions of hypoxia. The target cell may be a growth-arrested cell capable of undergoing cell division such as a cell in a central portion of a solid tumour mass or a stem cell such as an HSC or a $CD34^+$ cell. As a further alternative, the target cell may be a precursor of a differentiated cell such as a monocyte precursor, a $CD33^+$ cell, or a myeloid precursor. The target cell may also be a differentiated cell such as a neuron, astrocyte, glial cell, microglial cell, macrophage, monocyte, epithelial cell, endothelial cell or hepatocyte. Target cells may be transfected or transduced either *in vitro* after isolation from an individual or may be transfected or transduced directly *in vivo*.

Preferred host cells/target cells include cells in which EPAS is expressed, more preferably cells in which EPAS is expressed but HIF-1 is not (or at much lower levels).

In a particularly preferred embodiment, haematopoietic stem cells such as macrophages are used as host cells/target cells. As previously mentioned HSCs are pluripotent stem cells that give rise to all blood cell lineages in mammals. HSCs differentiate into various cell lineages under the influence of microenvironmental factors such as cell-to-cell interactions and the presence of soluble cell cytokines. Four major cell lineages arise from the HSCs. These include: erythroid (erythrocytes); megakaryocytic (platelets); myeloid (granulocytes and monocytes); and lymphoid (lymphocytes). Maturati n of these cells occurs under the influence of a network of tissue specific protein regulators

which have been given a variety of names including growth factors, cytokines and interleukins.

Macrophages, derived from monocytes from the bloodstream, have been used as a delivery vehicle for targeting drugs and therapeutic genes to solid tumours. It has been shown that macrophages continually enter solid tumours and congregate in poorly vascularised, ischaemic sites in breast carcinomas. Moreover, the degree of ischaemia-induced necrosis in these tumours was positively correlated with the degree of intra-tumoral macrophage infiltration.

Monocytes and macrophages also infiltrate ischaemic lesions which are a feature of other disease states including cerebral malaria, coronary heart disease and rheumatoid arthritis. Thus monocytes and macrophages are suitable host cells for use in the present invention. In particular, monocytes and/or macrophages comprising polynucleotides and/or vectors, such as viral vectors, of the invention are suitable for use in *ex vivo* and *in vivo* methods for treating diseases associated with hypoxia.

Methods for isolation of HSCs and their maintenance and differentiation in culture are known in the art (Santiago-Schwartz *et al* 1992; Charbord *et al.*, 1996; Dao *et al.*, 1997; Piacibello *et al.*, 1997) and in WO-A-91/09938. Retroviral gene transfer into human HSCs in the general sense has been reported (Duphar and Emmons, 1994). Methods for retroviral mediated transduction of HSCs and transfer to patients are also described by Dunbar *et al.*, 1996.

In vivo murine studies have indicated that the pretreatment of donor mice with 5-fluorouracil prior to harvest of bone marrow can improve transduction efficiencies by inducing the cycling of primitive cells and increasing the susceptibility to retroviral infection and integration. The co-culture of target cells with a retroviral producer cell line and the use of cell lines capable of producing at least 10^5 viral particles per ml has also improved efficiencies (Bodine *et al.*, 1991). Successful gene transfer into long term re-populating cells has been achieved in virtually all recipient mice with

reconstitution of multiple haematopoietic lineages stably with 1-50% or more cells carrying the proviral genome (Fraser *et al.*, 1990).

Where the invention uses a vector for delivery of a nucleic acid sequence to HSCs *in vivo*, the vector is preferably a targeted vector capable of targeting CD34+ HSCs.

The term "targeted vector" refers to a vector whose ability to infect/transfect a cell or to be expressed in the target cell is restricted to certain cell types within the host organism, usually cells having a common or similar phenotype. An example of a targeted vector is a targeted retroviral vector with a genetically modified envelope protein which binds to cell surface molecules found only on a limited number of cell types in the host organism. Another example of a targeted vector is one which contains promoter and/or enhancer elements which only permit expression of one or more retroviral transcripts in a proportion of the cell types of the host organism. Thus, the vector may be provided with a ligand specific for CD34, such as an antibody or an immunoglobulin-like molecule directed against CD34. On introduction into an individual to be treated such a vector will specifically transfect CD34+ HSCs. The vector may be administered systemically, to the peripheral circulation.

THERAPEUTIC USES

The present invention is believed to have a wide therapeutic applicability - depending on *inter alia* the selection of the one or more NOIs, prodrug activating domains and/or prodrugs. In addition, or in the alternative, the present invention may be useful in the treatment of disorders listed in WO-A-98/09985. For ease of reference, part of that list is now provided: macrophage inhibitory and/or T cell inhibitory activity and thus, anti-inflammatory activity; anti-immune activity, i.e. inhibitory effects against a cellular and/or humoral immune response, including a response not associated with inflammation; inhibit the ability of macrophages and T cells to adhere to extracellular matrix components and fibronectin, as well as up-regulated fas receptor expression in T cells; inhibit unwanted immune reactions and inflammation including arthritis,

including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reacti ns, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididimo-orchitis, infertility, orchidal trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fondus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillaim-Barre syndrome, Sydenham chora, myasthenia gravis, pseudo-tumour cerebri,

Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery, bone marrow transplantation or other transplantation complications and/or side effects, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers, natural or artificial skin tissue.

In particular, polynucleotides, nucleic acid vectors, viral vectors and host cells of the present invention may be used in the treatment of tumours. Examples of tumours that may be treated by the present invention include but are not limited to: sarcomas including osteogenic and soft tissue sarcomas, carcinomas such as breast, lung, bladder, thyroid, prostate, colon, rectum, pancreas, stomach, liver, uterine, and ovarian carcinoma, lymphomas including Hodgkin and non-Hodgkin lymphomas, neuroblastoma, melanoma, myeloma, Wilms tumour, and leukemias, including acute lymphoblastic leukemia and acute myeloblastic leukemia, gliomas and retinblastomas.

A particularly advantageous feature of the present invention is that nucleic acid sequencs may be expressed in hypoxic cells and not cells under normoxic conditions. Thus nucleic acid sequences, nucleic acid vectors, viral vectors and host cells of the present invention may be used in the clinical management of a range of conditions characterised by hypoxia. Example of conditions which are characterised by symptoms of hypoxia include stroke, deep vein thrombosis, pulm nary embolus and renal failure. The cell death of cardiac tissue, called myocardial infarction, is due in large part to

tissue damage caused by ischemia and/or ischemia followed by reperfusion. Other examples include cerebral malaria and rheumatoid arthritis.

It is especially preferred to use nucleic acid sequences, nucleic acid vectors, viral vectors and host cells of the present invention in the clinical management of solid tumours such as ovarian tumours, in particular tumours comprising tumour cells under hypoxic conditions. Treatment may effect a slowdown in the rate of tumour growth, a cessation in the rate of tumour growth or indeed shrinkage of tumour mass without necessarily resulting in complete apoptotic/necrotic death of all malignant cells in an affected patient.

The nucleic acid sequences, nucleic acid vectors, viral vectors and host cells of the present invention may also be used in preventative medicine. Thus, e.g. the prodrugs used in the invention may have a therapeutic effect via prophylaxis. For example, where an increased risk of developing cancer is diagnosed, the invention may be used to vaccinate the at-risk individual.

Suitability for prophylaxis may be based on genetic predisposition to cancer, for example cancer of the breast or ovary because of one or more mutations in a BRCA-1 gene, a BRCA-2 gene (Cornelisse *et al.*, 1996) or another relevant gene.

ADMINISTRATION

The nucleic acid sequences, nucleic acid vectors and viral vectors of the invention may thus be used to deliver therapeutic genes to a human or animal in need of treatment.

The nucleic acid sequences of the invention may be administered directly as a naked nucleic acid construct, preferably further comprising flanking sequences homologous to the host cell genome. Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known techniques including biolistic transformation and lipofection. Alternatively, the nucleic acid sequences may be administered as part of a

nucleic acid vector, including a plasmid vector or viral vector, preferably a lentiviral vector.

Preferably the delivery vehicle (i.e. naked nucleic acid construct or viral vector comprising the polynucleotide for example) is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Thus, the present invention also provides a pharmaceutical composition for treating an individual by gene therapy, wherein the composition comprises a therapeutically effective amount of the nucleic acid sequences, vector or viral vector of the present invention comprising one or more deliverable therapeutic and/or diagnostic NOI(s) or a viral particle produced by or obtained from same, together with a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The pharmaceutical composition may be for human or animal usage.

The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s), and other carrier agents that may aid or increase the viral entry into the target site (such as for example a lipid delivery system).

The pharmaceutical composition may be formulated for parenteral, intramuscular, intravenous, intracranial, subcutaneous, intraocular or transdermal administration.

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone r in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected

parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The pharmaceutical composition is administered in such a way that the nucleic acid sequences/vector containing the therapeutic gene for gene therapy, can be incorporated into cells at an appropriate area.

The active ingredient in such compositions may comprise from about 0.1% to about 99% by weight of the formulation. By "pharmaceutically acceptable" is meant that the ingredient must be compatible with other ingredients of the compositions as well as physiologically acceptable to the patient.

Pharmaceutical compositions for use according to the present invention may be formulated in conventional manner using readily available pharmaceutical or veterinary aids. Thus the active ingredient may be incorporated, optionally together with other active substances, with one or more conventional carriers, diluents and/or excipients, to produce conventional galenic preparations such as tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols, soft and hard gelatin capsules, suppositories, sterile injectable solutions, sterile packaged powders, and the like.

Examples of suitable carriers, excipients, and diluents are lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, aglinates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water syrup water, water/ethanol, water/glycol, water/polyethylene, glycol, propylene glycol, methyl cellulose, methylhydroxybenzoates, propyl hydroxybenzoates, talc, magnesium stearate, mineral oil or fatty substances such as hard fat or suitable mixtures

thereof. The compositions may additionally include lubricating agents, wetting agents, emulsifying agents, suspending agents, preserving agents, sweetening agents, flavouring agents, and the like. The formulations may be formulated so as to provide quick, sustained or delayed release of the active ingredient after adminstration to the patient by use of procedures well known in the art.

The compositions are preferably formulated in a unit dosage form, e.g. with each dosage containing from about 0.1 to about 500 mg of the active ingredient.

The precise dosage of the active ingredient and the length of the treatment will depend upon a number of factors including the age and weight of the patient, the specific condition being treated and its severity, and the route of administration. In general, an effective dose will be of the order of from about 0.01 mg/kg to about 20 mg/kg bodyweight per day, e.g. from about 0.05 to about 10 mg/kg per day, administered one or more times daily. Thus, an appropriate dose for an adult may be from 10 to 100 mg per day, e.g. 20 to 50 mg per day.

Administration may be by any suitable method known in the art, including for example oral, parenteral (e.g. intramuscular, subcutaneous, intraperitoneal or intravenous), rectal or topical administration.

When the nucleic acid sequences of the invention is delivered to cells by a viral vector of the invention, the amount of virus administered is in the range of from 10^3 to 10^{10} pfu, preferably from 10^5 to 10^8 pfu, more preferably from 10^6 to 10^7 pfu. When injected, typically 1-10 ml of virus in a pharmaceutically acceptable suitable carrier or diluent is administered.

When the nucleic acid sequence/vector is administered as a naked nucleic acid, the amount of nucleic acid administered is typically in the range of from 1 μg to 10 mg, preferably from 100 μg to 1 mg.

Where the NOI is under the control of an inducible regulatory sequence, it may only be necessary to induce gene expression for the duration of the treatment. Once the condition has been treated, the inducer is removed and expression of the NOI is stopped. This will clearly have clinical advantages. Such a system may, for example, involve administering the antibiotic tetracycline, to activate gene expression via its effect on the tet repressor/VP16 fusion protein. Another system will involve the use of desferrioxamine to induce HRE.

The use of tissue-specific promoters will be of assistance in the treatment of disease using the polynucleotides/vectors of the invention. For example, several neurological disorders are due to aberrant expression of particular gene products in only a small subset of cells. It will be advantageous to be able express therapeutic genes in only the relevant affected cell types, especially where such genes are toxic when expressed in other cell types.

Modified HSCs of the invention are administered to a patient or an at-risk individual in a suitable formulation. The formulation may include an isotonic saline solution, a buffered saline solution or a tissue-culture medium. The cells are administered by bolus injection or by infusion intravenously or directly to the site of a tumour or to the bone marrow at a concentration of for example between approximately 10^6 and of the order of 10^{12} cells / dose, preferably at least 10^8 or 10^{10} cells per dose.

The individual may first be treated to deplete the bone marrow of stem cells or may be treated with one or more cytokines such as G-CSF to increase the mobilisation of stem cells into the peripheral blood or one or more cytokines to enhance repopulation of bone marrow. Combinations of such treatments are also envisaged. The treatments of the invention may also be combined with currently available anti-cancer therapies.

In the event that the vector used for stem cell engineering encodes a prodrug activating enzyme, the individual suffering from cancer is additionally treated with the

corresponding prodrug, administered using an appropriate regimen according to principles known in the art.

The individual may also be administered with a bioreductive moiety according to principles known in the art.

Where the HSCs are removed from the individual to be treated and are transfected or transduced with the vector *in vitro*, the cells are generally expanded in culture prior to and after introduction of the NOI or NOIs. When cultured *in vitro* under appropriate conditions or when appropriate signals are received *in vivo*, HSC have the capacity to differentiate into, among other cell types, endothelial cells, myeloid cells, dendritic cells and immune effector cells such as neutrophils, lymphocytes, mononuclear phagocytes and NK cells.

This involves the use of tissue culture methods which are known in the art and include exposure to cytokines and/or growth factors for the maintenance of HSCs (Santiago-Schwartz *et al.*, 1992; Charbord *et al.*, 1996; Dao *et al.*, 1997; Piacibello *et al.*, 1997). Agents which induce the differentiation of the HSCs may also be added.

In addition to responding to hypoxia the HRE elements are known to respond to chemical inducers that mimic hypoxia. Two of these are known, these are cobalt chloride and desferrioxamine (Meliillo *et al.*, 1996; Wang and Semenza 1993b). Thus, the products of the invention where HRE is used may also be used to treat a disorder where compounds that mimic hypoxia are administered, such as the chemical activator desferrioxamine or analogous chemicals used to treat neuroblastoma (Blatt, 1994), beta thalassemia (Giardina and Grady, 1995), Alzheimers disease (Crapper *et al.*, 1991), VEGF deficiency (Beerrepoot *et al.*, 1996), Erythropoetin deficiency (Wang and Semenza, 1993b) and for enhancement of tumour chemotherapy (Voest *et al.*, 1993). The products of the invention may be adminstered concomitantly, sequentially or separately with the compounds that mimic hypoxia.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the ptimum route of administration and dosage for any particular patient and condition.

Suitability for prophylaxis may be based on genetic predisposition to cancer, for example cancer of the breast or ovary because of one or more mutations in a BRCA-1 gene, a BRCA-2 gene (Cornelisse *et al* 1996 Pathol Res Pract 192: 684-693) or another relevant gene.

It is now recognised that it may be important to identify patients in which a particular product will be safe and effective. This is based on the recognition that genetic variations may contribute to the variable effects of drugs in different individuals.

Thus, the present invention provides a method of testing an individual's DNA for polymorphisms in drug-metabolizing enzymes and then administering the prodrug activating agent of the present invention if appropriate.

In accordance with the invention, standard molecular biology techniques may be used which are within the level of skill in the art. Such techniques are fully described in the literature. See for example; Sambrook *et al* (1989) Molecular Cloning; a laboratory manual; Hames and Glover (1985 - 1997) DNA Cloning: a practical approach, Volumes I- IV (second edition); Methods for the engineering of immunoglobulin genes are given in McCafferty *et al* (1996) "Antibody Engineering: A Practical Approach".

It should be appeciated that features from various sections, aspects and embodiments of the invention as described above are generally equally applicable to other sections, aspects and embodiments *mutatis mutandis*.

The invention will now be described in further details with reference to the following non-limiting examples:

While the invention is exemplified with reference to the use f the human NADPH:cytochrome c reductase in gene therapy it can be applied to any P450 reductase derived from any species and modified to improve enzyme activity by the methods of protein engineering and to any other natural, genetically engineered or synthetic prodrug activating enzymes, for example, DT diaphorase, thymidine kinase, E.coli nitroreductase and Carboxypeptidase G_2.

Example 1:The addition of nuclear targeting peptides to P450R. [Figure 7.1]

Peptides or protein fusions are prepared by making hybrid DNA molecules using standard procedures known to one ordinarily skilled in the art of recombinant DNA technology. For example as described in the literature e.g. Sambrook *et al* 1989 Molecular Cloning: A Laboratory Approach; Hames and Glover 1985-1997 DNA cloning: a practical approach Volumes I-IV (Second Edition). The hybrid genes are inserted into suitable vectors for transfer to mammalian cells. There are many types of vector available which have been described in detail (e.g. Lever and Goodfellow 1995, Brit. Med. Bull. 51, Number 1, Gene Therapy) and which will be known to ordinary practitioners. For example, for production of a retroviral vector capable of expressing the prodrug activating enzymes described in this and subsequent examples the relevant coding region is inserted into a retroviral vector in which transcripts containing the P450 coding sequences are produced from a strong promoter such as the hCMV-MIE promoter. A suitable plasmid is pHIT111 (Soneoka *et al.* 1995 Nucl. Acids Res. 23; 628-633) and the required gene is inserted in place of the LacZ gene using standard techniques. The resulting plasmid is then transfected into the FLYRD18 or FLYA13 packaging cell lines (Cosset *et al.* 1995 J. Virol. 69; 7430-7436) and transfectants are selected for resistance to G418 at 1 mg/ml. G418-resistant packaging cells produce high titres of recombinant retrovirus capable of infecting human cells. The virus preparation is then used to infect human cancer cells and can be injected into tumours *in vivo*. The prodrug activating enzyme is then expressed from the tumour cells.

In pHIT111, the MoMLV LTR promoter-enhancer is used for expression of the therapeutic gene in the target cell. The vector can also be modified so that the therapeutic gene is transcribed from an internal promoter-enhancer such as one which is active predominantly in the tumour cells or one which contains a hypoxia regulated element. A suitable HRE-containing enhancer consists of a truncated HSV TK promoter with 3 copies of the mouse PGK HRE (Firth *et al.* 1994 Proc. Natl. Acad. Sci. 91: 6496-6500). The synthetic oligonucleotides described in eg WO95/21927 are inserted between the *Nhe1* and *Xba1* sites in the 3'LTR of pHIT111 (Soneoka *et al* ibid.) to generate a retroviral vector in which gene expression in the target cell is under hypoxia control. An alternative retroviral vector, constructed in the same way, is pKAHRE shown in Figure 6a. Other vector backbones which can be used are shown in Figure 6b.

In order to manipulate and analyse the coding sequence of the P450R gene it is subcloned into a mammalian cell expression vector as follows:-

Sequence ID NO: 1. Human P450R cDNA (Shephard *et al* 1992, Arch. Biochem. Biophy. 294:168, GenBank accession number : S90469, see Figure 1a).

The P450 reductase cDNA is obtained from human placental cDNA by conventional PCR amplification as an *EcoRI* fragment and ligated into the pBlueScript II vector to form P450R.1

In order to construct a nuclear targeted derivative of P450 reductase the ER anchor domain is removed to produce anchorless P450 reductase (alP450) and a nuclear localisation signal is added.

The functional domains of P450 reductase are shown in Figure 1b

SEQ ID NO:. The alP450R sequence is show in Figure 2A.

Any derivative of P450 reductase (alP450R, see SEQ ID NO: 3 figure 2a for the sequence) that has the SV40NLS substituted for the ER domain is obtained by PCR amplification from the plasmid pP450R.1 using the following primers :

NLS-alP450 5'-primer: SEQ ID NO: 7.

cccgccgcca ccatgCCAAA AAAGAAGAGA AAGGTA**TCCT CTGTCAGAGA**

GAGCAGCTTT G (61mer)

This contains the following components:

1. The Kozak leader sequence (Peakman *et al* 1992, Patent publication number EP0486170-A/2, GenBank accession number A18727). Lower case underlined.

2. The SV40 Large T antigen nuclear import signal sequence (Fiers *et al* 1978, Nature 273:113, see also Nigg 1997, Nature 386:779, GenBank accession number: V01380.

3. The 5' region of alP450R, (Smith *et al* 1994, PNAS 91:8710 Figure 3A).

alP450 3'-primer: SEQ ID NO: 8. The 3'-region of alP450R

CTAGCTCCAC ACGTCCAGGG AGTAG (25mer)

The resulting PCR fragment is subcloned into the *SmaI* site of the multiple cloning region of the pCIneo Vector (from Promega).

SEQ ID NO: 9. Multiple cloning site of pCINeo

gctagctcga gaattcacgcg tggtacctct agagtcga**CC CGGG**cggccg c
(NheI-XhoI-EcoRI-MluI-XbaI-AccI/SalI-**SmaI**-NotI)

The final fusion configuration is shown in Figure 7.1 .

Example 2. The addition f nuclear targeting peptides to the functional domain (FN) of P450R. [Figure 7.2]

The functional fragment of P450 reductase (FN fragment; (Smith *et al* 1994, PNAS 91:8710) is shown in Figure 2B. A nuclear targeted FN is constructed as follows:-

A PCR fragment of DNA sequence is produced that encodes a fusion protein of :-

SEQ ID NOS: 10 and 11. The SV40NLS (Figure 3)

SEQ ID NO: 5 and 6. The FN fragment of P450 reductase The sequence of P450 reductase FN fragment is shown in Figure 2B.

The PCR fragment is amplified from the pP450R.1 (Example 1) using the following primers :

FN 5'- primer: SEQ ID NO: 12. This contains the Kozak translation initiation sequence lower case, the SV40NLS upper case and the 5'-region of the FN fragment shown as bold, underlined in the sequence below:

ccgccgcca ccatg C CAAAAAAGAA GAGAAAGGTA**C GCCAGTACGA GCTTGTGGTC CACA** (60mer)

FN 3'-Primer: SEQ ID NO: 13. The 3'-coding region of the FN fragment

CTAGCTCCAC ACGTCCAGGG AGTAG (24mer)

The PCR fragment is subcloned into the *SmaI* site of the pCIneo Vector to produce SV40NLS-FN (Figure 7.2)

Example 3. Construction of a bFGF-alP450R expression vector [Figure 7.3]

Fusions are prepared as described for Example 1.

The 18kD isoform of bFGF (Prats *et al* 1989, PNAS 86:1836, see Figure 4B ; GenBank accession number: J04513).

A hybrid protein is constructed comprising at the N-terminus of the 18kD isoform of bFGF and at the C-terminus the anchorless P450 reductase (Figure 2A). This is done using two PCR reactions to lift the bFGF sequences from a bFGF-pUC18 plasmid (obtained from R & D systems) to produce a bFGF fragment and to lift the alP450R sequence from p450R.1 The two fragments are then digested with *SalI* and ligated together. The following primers are used:-

bFGF 5'-Primer: SEQ ID NO: 14. This primes the 5'-coding region of the 18kD isoform with the Kozak leader sequence (underlined).

cccgccgcca ccatggcagc cgggagcatc accacg (36mer)

bFGF 3'-Primer: SEQ ID NO: 15 . This primes the 3'-coding region of bFGF with a leading *SalI* restriction site (shown in upper case)

ggcgGTCGACgct cttagcagac attggaaga (32mer)

alP450R 5'-Primer: SEQ ID NO: 16 . This primes the 5'-coding region of alP450R with a leading *SalI* restriction site (shown in upper case)

ggcgGTCGAC tcc tctgtcagag agagcagctt tg (35mer)

alP450R 3'-Primer: SEQ ID NO: 17. This primes the 3'-coding region of alP450R.

ctagctccac acgtccaggg agtag (24mer)

The two PCR fragments bFGF and alP450R are then digested with *SalI*, purified and subsequently ligated to each other. The ligated DNA is then subcloned into the *SmaI* site of the mammalian expression vector pCIneo (Figure 7.3).

In some cases it is preferable to separate domains of proteins by a flexible linker. For example, but not restricted to, the sequence $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ (Somia *et al* 1993 PNAS 90, 7889). This is a procedure familiar to persons ordinarily skilled in the art of recombinant DNA technology and protein expression.

For example in order to place a flexible linker (flexlink) between the junctions of TTS and alP450 or FN protein domains an alternative set of PCR primers is used to create the following fragments:

Primer SalI-flexlink –alP450R: SEQ ID NO: 18. Sequences in bold represent rare codons in highly expressed genes (Haas *et al* 1996 Curr. Biol., 6, 315). Sequences underlined are the 5' regions of alP450R.

5'- ggcgGTCGAC **gga ggt gga ggt tcg** GGC GGG GGC GGC AGT GGG GGC GGC GGG AGT tcc tctgtcagag agagcagctt tg

Example 4 : Construction of bFGF-FN expression vector [Figure 7.4]

A cDNA fragment which encodes a fusion protein product of the 18 kD bFGF protein (see Figure 3B) and the FN domain (Figure 2B) is obtained by PCR amplification from the bFGF/PUC18 plasmid (R&D systems) the FN fragment is obtained from the plasmid pP450R.1 using the following primers :

bFGF primers: For the bFGF 5'- and 3'-primers see Example 3.

FN 5'-cPrimer : SEQ ID NO: 21. This primes the 5'-coding region of FN with a

leading *SalI* restriction site

ggcgGTCGAC cgc cagtacgagc ttgtggtcca ca (35mer)

FN 3'-Primer: SEQ ID NO:13. The 3'-coding region of the FN fragment

CTAGCTCCAC ACGTCCAGGG AGTAG (24mer)

The two PCR fragments bFGF and FN are then digested with *SalI*, purified and subsequently ligated to each other. The ligated DNA is then subcloned into the *SmaI* site of the mammalian expression vector pCIneo (Figure 7.4).

To place a flexible linke between the bFGF and FN domains an alternative 5' primer is used:-

Primer SalI-flexlink-FN: SEQ ID NO: 22

5'-ggcgGTCGAC **gga ggt gga ggt tcg** GGC GGG GGC GGC AGT GGG GGC GGC GGG AGT cgc cagtacgagc ttgtggtcca ca

Sequences in bold represent rare codons in highly expressed genes (Haas *et al* 1996 Curr. Biol., 6, 315). Sequences underlined are the 5' regions of FN.

Example 5 : Construction of an pAntp-alP450R expression vector [Figure 7.5]

SEQ ID NOS: 53 and 54. The *Drosophila melanogaster* antennapedia protein homeobox peptide (pAntp, Laughon *et al* 1986 Mol. Cell. Biol. 6:4676)

A cDNA fragment which encodes a fusion protein product of pAntp with alP450R is btained by PCR amplification of pAntp from plasmid p903G (Joliot *et al* 1991 PNAS

88:1864) and amplification of anchorless P450R from the plasmid pP450R.1 using the following primers :

pAntp 5'-Primer: SEQ ID NO: 23. This primes the 5'-coding region of pAntp) with a Kozak leading sequence (underlined).

cccgccgcca ccatggaacg caaacgcgga aggcagacat (40mer)

pAntp 3'-Primer: SEQ ID NO: 24. This primes the 3'-coding region with a *SalI* restriction site

ggcgGTCGACgtt ctccttcttc cacttcatgc gccga (38mer)

alP450R 5'-Primer: SEQ ID NO: 16. This primes the 5'-coding region with a leading *SalI* restriction site

ggcgGTCGACtcc tctgtcagag agagcagctt tg (35mer)

alP450R 3'-Primer: Seq ID no 17. This primes the 3'-coding region

ctagctccac acgtccaggg agtag

The two PCR fragments pAntp and alP450R are then digested with *SalI*, purified and subsequently ligated to each other. The ligated DNA is then subcloned into the *SmaI* site of the mammalian expression vector pCIneo (Figure 8.5).

Alternative primers can be used to insert flexible linkers as described in the preceding examples.

To enhance the secretion of pAntp fusions from cells a secretion signal can be added to the N-terminus. A suitable signal is derived from the 5T4 single chain antibody sequence (Figure 3F).

PAntp secretion 5'primer: SEQ ID NO: 28. This places a Kozak translation initation site and a secretion signal at the 5' end of the pAntp peptide. Sequences corresponding to pAntp are in upper case, Kozak initiation sequence is underlined.

ccaccatgggatggagctgtatcatcctcttcttggtagcaacagctacaggtgtccactccaaacgcggaaggcagaca
GAACGCAAACGCGGAAGGCAGACAT (105mer)

Example 6 : Construction of a pAntp-FN expression vector [Figure 7.6]

As in Example 5, a cDNA fragment which encodes a fusion protein product of the pAntp is obtained by PCR amplification from a pAntp containing plasmid e.g. p903G. The FN fragment (see Figure 2B) is obtained from the plasmid pP450R.1 using the following primers :

For the 5'-Primer and 3'-Primer of pAntp, see Example 5
For the 5'-Primer and 3'-primer of FN, Example 4.

The two PCR fragments pAntp and FN are then digested with *SalI*, purified and subsequently ligated to each other. The ligated DNA is then subcloned into the *SmaI* site of the mammalian expression vector pCIneo (Figure 7.6). Alternatively the pAntp secretion 5' primer, SEQ ID NO: 28 can be used.

Example 7 : Construction of a VP-alP450R expression vector [Figure 7.7]

SEQ ID NOS: 55 and 56 . The Herpes Simplex Virus Type-1 tegument protein VP22 (Elliott and O'Hare 1997 Cell 88:223, GenBank accession number : X14112)

A fusion protein product of VP22 fused to alP450R is obtained by PCR amplification from a VP-22 containing plasmid, pGE109 and by PCR amplification from the plasmid pP450R.1 using the following primers:

VP22 5' primer: SEQ ID NO:29. This primes the 5'-coding with a Kozak leading sequence (underlined).

cccgccgcca ccatgacctc tcgccgctcc gtgaag (36mer)

VP-22 3'-Primer: Seq. ID no. 30. This primes the 3'-coding region and contains a *SalI* restriction site.

ggcgGTCGACctc gacgggccgt ctggggcgag a (34mer)

For the 5'- and 3'-primers of alP450R, see Example 3.

The two PCR fragments VP-22 and alP450R are then digested with *SalI*, purified and subsequently ligated to each other. The ligated DNA is then subcloned into the *SmaI* site of the mammalian expression vector pCIneo (Figure 7.7).

Example 8 : Construction of an VP-FN expression vector [Figure 7.8]

As in Example 7, a cDNA fragment which encodes a fusion protein product of the VP-22 (see Figure 3D) is obtained by PCR amplification from pGE109 and the FN fragment (see Figure 2B) is obtained from the plasmid pP450R.1 using the following primers :

For the VP22 5'- and 3'-primers, see Example 7

For the FN 5'-and 3'-primers, see Example 4

The two PCR fragments pAntp and FN are then digested with *SalI*, purified and subsequently ligated to each other. The ligated DNA is then subcloned into the *SmaI* site of the mammalian expression vector pCIneo [Figure 7.8].

Example 9A. Construction of a 5T4scFv-PEA-alP450R expression vector [Figure 7.9]

The construction of this fusion protein involves the PCR amplification and then ligation of three DNA fragments to form the full insert. The first fragment which encodes a protein product of a single chain variable antibody fragment against the tumour antigen 5T4.

SEQ ID NO: 26. The coding sequence of 5T4 scFv Figure 3F. This is obtained by PCR amplification from a convenient plasmid, pPs-5T4, containing this sequence using the following primers :

Fragment 1: 5T4 scFv

5T4scFv 5'-primer: SEQ ID NO: 31. This primer provides the translation initiation signal and the secretion signal region from the 5T4scFv sequence (Figure 3F)

ccaccatggg atggagctgt atca (24mer)

5T4scFv 3'-primer: SEQ ID NO: 32. This primes the 3'-region of the 5T4 scFv fragment with a *HindIII* restriction site

ggccAAGCTTccg tttgatttcca gcttggag (32mer)

Fragment 2 PEA fragment.

SEQ ID NOS: 33 and 34. The protein of the domain II of Pseudomonas aeruginosa exotoxin A (PEA, GenBank accession number K01397 and M23348) (Figure 3E). This is obtained as a PCR fragment using the following primers

PEA 5'-primer : SEQ ID NO: 35. This primes the 5'-coding region with a *HindIII* restriction site

ggcgAAGCTTggc agcctggccg cgctgaccgcg ca (35mer)

PEA 3'-primer: SEQ ID NO: 36. This primes the 3'-coding region with a *EcoRI* restriction site

ggcgGAATTCgtt ggccgcgccc cggtcgtcgt t (34mer)

Fragment 3. alP450R

The third cDNA fragment encoding alP450R (see Figure 2A) is obtained by PCR amplification from the plasmid pP450R.1 using the following primers :

alP450R 5'-primer: SEQ ID NO: 37.This primes the 5'-coding region with a *EcoRI* site

ggcgGAATTCtca gctcttagca gacattggaa g (34mer)

alP450R 3'-primers: As in Example 3 Seq. ID No 17.

The first and the second PCR fragments are digested with *HindIII* and then ligated together. Then the ligate and the third PCR fragment are digested with *EcoRI* and ligated to form the full insert. The full insert is subcloned into the *SmaI* site of the pCIneo expression vector (figure 7.9).

Example 9B : Construction f a 5T4scFv-alP450R-MTS expression vector [Figure 7.13]

The construction of this fusion protein requires the PCR amplification and subsequent ligation of three DNA fragments to form the full insert. The first fragment encodes a protein product of a single chain variable antibody fragment against the tumour antigen 5T4, as in Example 9A.

SEQ ID NO: 26. The coding sequence of 5T4scFv Figure 3F. This is obtained by PCR amplication from a convenient plasmid, pPs-5T4, containing this sequence using the following primers :

Fragment 1 : 5T4 scFv

5T4scFv 5'-primer : SEQ ID NO:31. This primer provides the the translation initiation signal and the secretion signal region from the 5T4scFv sequence (Figure 3F).

ccaccatggg atggagctgt atca (24mer)

5T4scFv 3'-primer : SEQ ID NO:32. This primers the 3'-region with a *HindIII* restriction site

ggccAAGCTTccg tttgatttcca gcttggag (32mer)

Fragment 2: alP450R

The second cDNA fragment encoding alP450R (see Figure 2A) is obtained by PCR amplification from the plasmid pP450R.1 using the following primers :

alP450R 5'-primer : SEQ ID NO:38. priming the 5'-coding region with a *HindIII* restriction site

ggccAAGCTTtca gctcttagca gacattggaa g (34mer)

alP450R 3'-Primer : SEQ ID NO:39 priming the 3'-coding region of alP450R with a *EcoRI* restriction site

gccgGAATTCgct ccacacgtcc agggagtag (32mer)

Fragment 3 : MTS

The third fragment encoding the membrane translocating sequence (MTS) is obtained by PCR amplification using the following primers :

MTS 5'-Primer : SEQ ID NO:40 primes the 5'-coding region with a *EcoRI* restriction site

gccgGAATTCgca gccgttcttc tccctgttct tcttgccgca ccc (46 mer)

MTS 3'-Primer : SEQ ID NO:41 primes the 3'-coding region

ttagggtgcg gcaagaagaa cagggagaag aacggctgc (39 mer)

The first and the second PCR fragments are digested with *HindIII* and then ligated together. Then the ligate and the third PCR fragment are digested with *EcoRI* and ligated to form the full insert. The full insert is subcloned into the *SmaI* site of the pCIneo expression vector. (Figure 7.13).

Example 10A : Construction of a 5T4scFv-PEA-FN expression vector [Figure 7.10]

The construction of this vector involves the PCR amplification and then ligation of three DNA fragments to form the insert. The first fragment, which encodes a protein product of a single chain variable antibody fragment against the tumour antigen 5T4, is obtained as in Example 9A.

The second cDNA fragment encoding a protein of the domain II of Pseudomonas aeruginosa exotoxin A (PEA, GenBank accession number K01397 and M23348) is obtained as in Example 9A.

PEA 5'-and 3'-primers are as in Example 9A.

The third cDNA fragment encoding FN (see Figure 2B) is obtained by PCR amplification from the plasmid pP450R.1 using the following primers :

FN 5'-primer: SEQ ID NO: 32. This primes the 5'-coding region with an *EcoRI* restriction site

ggcgGAATTCcgc cagtacgagc ttgtggtcca ca (35mer)

FN 3'-primers: These are as in Example 4.

The first and the second PCR fragments are digested with *HindIII* and then ligated together. The ligation product and the third PCR fragment are digested with *EcoRI* and ligated to form the full insert. The full insert is subcloned into the *SmaI* site of the pCIneo expression vector [Figure 7.10].

Example 10B : Construction of a 5T4scFv-FN-MTS expression vector

The construction of this vector involves the PCR amplification and then ligation of three DNA fragments to form the insert. The first fragment, which encodes a protein product

of a a single chain variable antibody fragment against the tumour antigen 5T4, is obtained as in Exampl 9A.

The second fragment encoding FN (see Figure 2B) is obtained by PCR amplification from the plasmid pP450R.1 using the follwing primers:

FN 5'-Primer : SEQ ID NO:43. This Primes the 5'-encoding region with a *HindIII* restriction site.

ggccAGGTTCcgc cagtacgagc ttgtggtcca ca (35 mer)

FN 3'-Primer : SEQ ID NO:44. This primes the 3'-encoding region with a *EcoRI* restriction site.

gccgGAATTCcta gctccacacg tccagggagt ag (35 mer)

The third fragment encoding MTS is obtained by PCR amplification as in Example 9B.

The first and the second PCR fragments are digested with *HindIII* and then ligated together. Then the ligate and the third PCR fragment are digested with *EcoRI* and ligated to form the full insert. The full insert is subcloned into the *SmaI* site of the pCIneo expression vector (Figure 7.14).

Example 11. Construction of a P4502B6/FN fusion protein [Figure 7.11]

SEQ ID NO: 33. The coding region for P4502B6 (Yamano *et al* 1989 Biochemistry, 28, 7340; GenBank Accession No. JO2864) (Figure 4). This is subcloned as an *NheI* to *XhoI* fragment into pCI-Neo using the following primers.

2B6 Nhe 5' primer: SEQ ID NO: 34

ggcgGCTAGC cagaccatggaactcagcg (29mer)

2B6 3'primer: SEQ ID NO: 35. This primer modifies the C-terminus of the protein to remove the stop codon and replace it with an *XhoI* site. The PCR fragment is digested with *NheI* and *XhoI* and sub-cloned into pCINeo to produce pCI-Neo containing a truncated P450.

ggcgCTCGAG gcggggcaggaagcggatctgg (32 mer)

A flexi-link FN fragment is constructed using the following primers

Flexi-FN 5' primer: SEQ ID NO: 36. This places a *SalI* restriction site followed by a flexible linker at the N-terminus of the FN fragment. Rare codons in the linker are bold lower case; sequences in FN are underlined. *SalI* site is upper case.

-ggcgGTCGAC **gga ggt gga ggt tcg** GGC GGG GGC GGC AGT GGG GGC GGC GGG AGT cgc cagtacgagc ttgtggtcca ca (80 mer)

FN 3' primer : As in Example 4.

CTAGCTCCAC ACGTCCAGGG AGTAG (24mer)

2B6 PCR fragment is digsted with *NheI* and *XhaI*, ligated to the *SalI* digested FN fragment, the fragment is purified and then digated into *XhoI/SmaI* digested pCIneo [Figure 7.11].

Example 12 : Construction of a double expression cassette for P450 and P450R [Figure 7.12]

P4502B6 is subcloned into pCINeo as an *NheI/XhoI* fragment using the following primers:

2B6 Nhe 5' primer: SEQ ID NO: 37

ggcgGCTAGC cagaccatggaactcagcg

2B6 terminus 3' primer: SEQ ID NO: 38. This primer retains the authentic translation stop signal

5' ggcgCTCGAG tcagcggggcaggaagcggatctgg

SEQ ID NO: 39. An internal ribosome entry site derived from FMDV R100 (IRES, Figure 5 Escarmis *et al* 1992 Virus Res. 26, 113).

This is inserted into the *XhoI* site of the plasmid pCI-Neo containing the 2B6 sequence as described above. The IRES is isolated as an *XhoI/SalI* fragment using the relevant 5' and 3' primers derived from the sequence shown in Figure 6. The resulting plasmid is digested with *SalI* and the *XhoI/SalI* fragment derived from P450R.1 is inserted. The final configuration is shown in Figure 7.12.

Example 13 : Construction of a double expression cassette for P450/FN and VP-FN.

Combinations of coding regions containing the active entity of P450 and P450R are constructed. One example is the coexpression of VP-FN with P450FN.

The fusion constructed described in Example 8 Figure 8.8 is inserted into the *HpaI* site of the retroviral vector COI (Figure 6b) and the fusion construct described in example 11 Figure 7.11 is inserted into the *XhoI* site of COI. This produces a vector that confers maximum sensitivity Cyclophosphamide, Tirapazamine and Mitomycin C. Figure 8.

Example 14B : The construction of retroviral vectors expressing modified prodrug activating enzymes.

RRVs are produced using a packaging cell line system such as FLYRD18 (Cosset *et al*). A plasmid vector containing the vector genome to be packaged is transfected into the packaging cell line as described (Cosset FL *et al*, 1995 J Virol 69 7430-7436) to derive the producer cell line. A suitable plasmid containing vector genome is pHIT111 (Soneoka Y *et al* 1995 Nucl Acids Res 23 628-633). The required therapeutic gene is inserted in place of the LacZ gene in pHIT111 using standard molecular biology techniques. Regulatory elements such as HREs and / or enhancer elements may similarly be introduced into the retroviral LTR in pHIT111 in place of the retroviral enhancer to ensure regulated expression of the therapeutic gene. The plasmid is then co-transfected with a selectable marker gene appropriate for FLYRD18 cells (eg pSV2neo) and transfected cells are selected in 1 mg/ml G418 (Sigma). An alternative vectors are PKAHRE which is an MLV based single transciption unit vector (Figure 6). Expression of therapeutic genes is controlled by a hypoxia responsive promoter (3xPGK Dachs *et al* op. cit.). The gene encoding the prodrug activating enzyme is substituted for the nlsLAcZ gene shown. An alternative vector that is particularly suitable for the expression of multiple transcription units is COI (Figure 6). This is a MLV based vector with the CMV enhancer replacing the MLV enhancer (shaded box). The gene encoding the prodrug activating enzyme is inserted either into the *Bam/Sal/Hpa* poly linker or the *Stu/Xho* polylinker. If necessary the ends of the fragments to be inserted are modified with suitable linkers to create the apropriate compatible restriction sites. Alternatively different genes can be inserted into each polylinker site (Figure 8).

Alternatively lentiviruses may be used and this is described in more detail in Example 14B.

Example 14B : Generation of EIAV vector genomes expressing P450

Construct details

peg HRElacZ is described in patent application No. 9901906.9 and is repeated bel w.

pEGASUS4 (also pEGASUS1), pONY4.0, pONY4.1, pONY3.1, pHORSE3.1 are described in PCT/GB98/03876 and are also described below.

OBhre1

Trimer encompassing –307/-290 sequence of murine PGK in the natural orientation (Firth *et al.*, 1995) linked to the SV40 promoter (italicised).

GCTAGAGTCGTGCAGGACGTGACATCTAGTGTCGTGCAGGACGTGACA
*Nhe*I HRE HRE
TCTAGTGTCGTGCAGGACGTGACAGCTAGCCCGGGCTCGAGATCTGCG
HRE *Xba*I
ATCTGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCC

CATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATCG

*CTGACTAATTTTTTTTATTTAT*GCAGAGGCCGAGGCCGCCTCGGCCTCTG
start start

This promoter sequence is defined as OBhre1. The resulting plasmid is designated plasmid OB37.

pEGASUS-4 (also pEGASUS-1)

The hypoxia responsive promoter has been configured into a lentiviral vector, pEGASUS-1 (also pEGASUS-4(+)) and the related vector pONY2.1. Both are derived from infectious proviral EIAV clone pSPEIAV19 (Payne *et al.*, 1998; Accession No. U01866). The construction of these plasmids is described below (taken from U.K. patent application no. 9727135.7).

pONY2.1

Plasmid pONY1 was constructed by inserting the EIAV 5' LTR into pBluescript II KS$^+$ (Stratagene). The EIAV 5' LTR was amplified by PCR from pSPEIAV19 using pfu polymerase and the following primers - 5' GCATGGACCTGTGGGGTTTTTATGA GG and 3' GCATGAGCTCTGTAGGATCTCGAACAGAC. The amplification product was blunt-ended by 5' overhang fill-in and inserted into pBluescript II KS$^+$ cut with *BssHII* which had been blunt ended by 3' verhang removal using T4 DNA polymerase

This construct was called pONY1 and the orientation was 5' to 3' in relation to β-galactosidase of pBluescript II KS$^+$. Sequencing of pONY1 revealed no mutations.

The *env* region of pSPEIAV19 was deleted by removal of the *Hind* III/*Hind* III fragment to generate pSPEIAV19ΔH. The MluI/MluI (216/8124) fragment of pSPEIAV19ΔH was then inserted into pONY1 cut with MluI to generate a wild type proviral clone (pONY2ΔH) in pBluescript II KS$^+$. A *BssHII* digest (619/792) of pBluescript II KS$^+$ was carried out to obtain the multiple cloning site. This was blunt ended by 5' overhang fill-in and ligated to pONY2ΔH cut with *BglII* and *NcoI* (1901/4949 - within *pol*) and blunt-ended by 5' overhang fill-in. The orientation was 3' to 5' in relation to the EIAV sequence. This was called pONY2.1.

pSPCMV was created by inserting pLNCX (Genbank Accession number: M28246) (*PstI/HindIII*) into pSP72 (Promega; Genbank Accession No. X65332). The β-galactosidase gene was inserted from pTIN414 (Cannon *et al.*, 1996) into pSPCMV (*XhoI/SphI*) to make pSPlacZ. The 5' end to the β-galactosidase gene was replaced by the SV40 T-antigen nuclear localization signal from pAD.RSVbgal (Stratford-Perricaudet *et* al., 1992) to make pSPnlslacZ (*XhoI/ClaI*). The CMV nuclear localizing and non-nuclear localizing β-galactosidase from pSPlacZ and pSPnlslacZ was cut out with *PstI* and inserted into the *PstI* site of pONY2.1 in the 5' to 3' orientation of EIAV. These were called pONY2.1nlslacZ and pONY2.1lacZ.

<u>pEGASUS-1</u>

This is an EIAV-based vector that contains only 759 nt of EIAV sequences (268 nt-675 nt and 7942 nt-8292 nt). Sequences encompassing the EIAV polypurine tract (PPT) and the 3'LTR were obtained by PCR amplification from pONY2.1 using primers PPTEIAV+ (Y8198): GACTACG<u>ACTAGT</u>GTATGTTTAGAAAAACAAGG, and 3'NEGSpeI (Y8199): CTAGGCT<u>ACTAGT</u>ACTGTAGGATCTCGAACAG. The product was purified, digested with *SpeI* and ligated into pBS II KS$^+$ which had been prepared by digestion with *SpeI* and treatment with alkaline phosphatase. Colonies

obtained following transformation into *E. coli*, XL-1Blue were screened for the presence of the 3'LTR in the orientation in which the U5 region of the 3'LTR was proximal to the *NotI* site of the pBS II KS^+ linker. The sequence of the cloned insert was determined and showed that it contained only one change from the EIAV clone pSPEIAV19. This was a 'C' insertion between bases 3 and 4 of the R region. The same change was found in the template used in the PCR reaction. The clone was termed pBS.3'LTR.

Next a reporter gene cassette, CMV promoter/LacZ, was introduced into the *PstI* site of pBS.3'LTR. The CMV/LacZ cassette was obtained as a PstI fragment from pONY2.1. The ligation reaction to join the above fragments was transformed into *E. coli*, XL-1Blue. A number of clones in which the CMV/LacZ insert was orientated so that the LacZ gene was proximal to the 3'LTR were assessed for activity of the CMV/LacZ cassette by transfection into the cell line 293T using standard procedures. A clone which gave blue cells at 48 hours post-transfection following development with X-gal was selected for further use and termed pBS CMVLacZ.3'LTR.

The 5' region of the EIAV vector was constructed in the expression vector pCI-ENeo which is a derivative of pCI-Neo (Promega; Genbank Accession No. U47120) modified by the inclusion of approximately 400 base pairs derived from the 5' end of the full CMV promoter as defined previously. This 400 base pair fragment was obtained by PCR amplifcation using primers VSAT1 (GGGCTATATGAGATCTTGAATAATAAAATG TGT) and VSAT2 (TATTAATAAC TAGT) and pHIT60 (Soneoka *et al.*, 1995) as template. The product was digested with *BglII* and *SpeI* and ligated into pCI-Neo which had been digested similarly.

A fragment of the EIAV genome running from the R region to nt 150 of the gag coding region (nt 268 to 675) was amplified with primers CMV5'EIAV2 (GCTAC GCAGAGCTCGTTTAGTGAACCGGGCACTCAGATTCTG: [sequences underlined anneal to the EIAV R region]) and 3'PSI.NEG

(GCTGAGCTCTAGAGTCCTTTTCTTT TACAAAGTTGG) using pSPEIAV19 as template DNA. The 5' region of the primer CMV5'EIAV2 contains the sequences immediately upstream of the CMV promoter transcriptional start site and can be cut with SacI (bold). 3'PSI.NEG binds 3' of the EIAV packaging sequences as defined by deletion analysis and contains an *XbaI* site.

The PCR product was trimmed with *SacI* and *XbaI* and ligated into pCI-Eneo which had been prepared for ligation by digestion with the same enzymes. This manipulation places the start of the EIAV R region at the transcriptional start point of the CMV promoter and transcripts produced thus start at the genuine start position used by EIAV and extend to the 3'-side of the packaging signal. Clones which appeared to be correct as assessed by restriction analysis were sequenced. A clone termed pCI-Eneo.5'EIAV was selected for further work.

In the next step the CMVLacZ and 3'LTR cassette in pBS.CMVLacZ.3'LTR was introduced into pCI-Eneo.5'EIAV. pBS.CMVLacZ.3'LTR was digested with *ApaI*, the 3'overhangs removed with T4 DNA polymerase, then digested with NotI. The fragment containing the CMVLacZ.3'LTR was purified by standard molecular biology techniques. The vector for ligation with this fragment was prepared from pCIEneo.5'EIAV by digestion with *SalI*, followed by filling-in of the 5'overhangs using T4 DNA polymerase. The DNA was then digested with NotI and purified prior to use in ligation reactions. Following transformation into *E.coli*, XL-1Blue colonies were screened for the presence of the insert by restriction analysis to identify the required clone, designated pEGASUS-1 (see Figure 21 which actually shows pEGASUS-4(+), a version of pEGASUS-1 containing an EIAV RRE immediately downstream of the EIAV R-U5-psi region - see below for details).

pEGASUS-4(+)

Further improvements to the EIAV vector pEGASUS-1 may be made by introduction of additional elements to improve titre. A convenient site for the introduction of such elements is the SalI site which lies between the XbaI to the 3' of th packaging signal

and upstream of the CMV/LacZ cassette of pEGASUS-1. For example the RRE from EIAV can be inserted at this site.

The EIAV RRE as defined previously was obtained by PCR amplification as follows. Using pONY2.10LacZ as template two amplifications were performed to obtain the two parts of the EIAV RRE. The 5'-element was obtained using primers ERRE1 (TTCTGTCGACGAATCCCAGGGGGAATCTCAAC) and ERRE2 (GTCACCTTCCAG AGGGCCCTGGCTAAGCATAACAG) and the 3'element with ERRE3 (CTGTTATG CTTAGCCAGGGCCCTCTGGAAGGTGAC) and ERRE4 (AATTGCTGACCCCCAAA ATAGCCATAAG). These products will anneal to each other hence can be used in second PCR reaction to obtain a DNA which 'encodes' the EIAV RRE. The second PCR amplification is set up without primers ERRE1 and ERRE4 for the first 10 cycles and then these primers are added to the reaction and a further 10 cycles of amplification carried out. The resulting PCR product and pEGASUS-1 were digested with SalI, ligated and transformed into E.coli XL-1Blue. Clones in which the EIAV RRE was in either the positive or negative orientations were selected for further work. These vectors plasmids were called pEGASUS-4(+) (see Figure 21) and pEGASUS-4(-).

pONY HRE luc/lac

The CMV promoter in pONY2.1 is excised as an *XbaI/AscI* fragment and replaced with an oligonucleotide containing a *MluI/XbaI* site. This consequently allows insertion of the MluI/XbaI fragment isolated from OB37 creating pONY HRE luc/lac. Luciferase coding sequence is removed as an NcoI fragment and the backbone religated creating pONY HRElac. Similarly, lacZ is removed as *XbaI/SalI* then the backbone religated to create pONY HREluc.

pEGHRELacZ/luc

The CMV promoter lacZ cassette is excised from the EIAV vector plasmid pEGASUS-1 with *EcoRI* and replaced with a synthetic oligonucleotide containing a SacI and a Bsu36 site. This allows the cloning of the HRE luc/HRE lac cassette from pONY HRE

luc and pONY HRE lac as *SacI/Bsu36* and *SacI/EcoR1* fragments respectively. The final vectors are designated pEG-HRE-lacZ and pEG-HRE-luc.

Using the same approach pEGHRE vectors may be constructed to express therapeutic genes, such as any of the therapeutic genes listed above, in place of the lacZ or Luc genes. These may be cloned into pEGHRE vectors by excision of the lacZ/luc fragments from pEG-HRE-lacZ or pEG-HRE-luc with *SacI/Bsu36* and *SacI/EcoR1*, respectively, and ligation of a suitable fragment containing a coding sequence. An example of a therapeutic gene which may be used is the gene encoding anti-angiogenic factor thrombospondin-1 (Genbank Accession No. X04665).

In order to construct a replication incompetent EIAV vector system we have used, as a starting point, an infectious proviral clone pSPEIAV19 (accession number: U01866), described by Payne et al. (1994, J Gen Virol. 75:425-9). An initial EIAV based vector was constructed by simply deleting part of *env* by removing a *Hind III/Hind III* fragment corresponding to coordinates 5835/6571 according to the numbering system of Payne et al. (ibid.). This fragment was replaced with the puromycin resistance gene under the control of the SV40 early promoter from pTIN500 (Cannon et al 1996 J. Virol. 70:8234-8240) to create pESP.

A further vector system was therefore constructed comprising three transcription units to produce the following: 1) vector genome RNA; 2) env and 3) gag-pol. In order to ensure that sufficient of each component is produced, the env and gag-pol transcription units are transcribed from a promoter-enhancer active in the chosen human packaging cell line. In this way, sufficient gag-pol and, most likely tat, are produced to ensure efficient production of transduction-competent vector particles.

The vector genome was constructed which has the reporter gene within the pol region of the genome as follows. The plasmid designated pONY1 was constructed by inserting the EIAV LTR, amplified by PCR from pSPEIAV19, into pBluescript II KS+

(Stratagene). The 5' LTR of EIAV clone pSPEIAV19 was PCR amplified using pfu polymerase with the following primers:

5' GCATGGACCTGTGGGGTTTTTATGAGG

3' GCATGAGCTCTGTAGGATCTCGAACAGAC

The amplicon was blunt ended by 5' overhang fill-in and inserted into pBluescript II KS+ cut with *Bss HII* which had been blunt ended by 3' overhand removal using T4 DNA polymerase. This construct was called pONY1 and the orientation was 5' to 3' in relation to β-galactosidase of pBluescript II KS+. Sequencing of pONY1 revealed no mutations.

The env region of pSEIAV19 was deleted at the *HindIII/HindIII* sites creating pSPEIAV19DH. Vector genome pSPEIAV19DH was cut with *Mlu I* (216/8124) and inserted into pONY1 *Mlu I* cut (216) to make pONY2. A *Bss HII* digest (619/792) of pBluescript II KS+ was carried out to obtain the multiple cloning site. This was blunt ended by 5' overhang fill-in and ligated to pONY2 cut with *Bgl II* and *Nco I* (1901/4949) and blunt ended by 5' overhang fill-in. The orientation was 3' to 5' in relation to the EIAV sequence. This was called pONY2.1. pSPCMV was created by inserting pLNCX (Accession number: M28246) (*Pst I/Hind III*) into pSP72 (Promega). The β-galactosidase gene was inserted from pTIN414 (Cannon PM et al J. Virol. 70, 8234-8240) into pSP72 (*Xho I/Sph I*) to make pSPlacZ. The 5' end to the β-galactosidase gene was replaced by the SV40 T antigen nuclear localization signal from pAD.RSVbgal (J. Clin. Invet. 90:626-630, 1992). pAD.RSVbgal was cut with *Xho I/ Cla I* and inserted into *Xho I/ Cla I* pSPlacZ to make pSPnlslacZ. The CMV nuclear localizing and non nuclear localizing β-galactosidase from pSPlacZ and pSPnlslacZ was cut out with Pst I and inserted into the Pst I site of pONY2.1 in the 5' to 3' orientation of EIAV. These were called pONY2.1nlslacZ and pONY2.1lacZ.

The gag-pol gene is expressed from the hCMV-MIE promoter-enhancer. In particular, gagpol pSPEIAV19DH was cut with *Mlu I* (216/8124) and inserted into pCI-Neo(Promega) *Mlu I* cut (216) to make pONY3.

The vector genome pONY2.1lacZ contains 1377nt of *gag*. RNA secondary structure prediction ("http://www.ibc.wustl.edu/~zuker/rna/") was used to identify possible stem-loop structures within the leader and the 5' end of gag. Based on these predictions four deletions were made within the gag region of pONY2.1lacZ. Deletions were made by PCR mutagenesis using standard techniques.

pONY2.11lacZ

pONY2.1lacZ contains 1377nt of *gag* (deleted from position 1901nt)

pONY2.11lacZ contains 354nt of *gag* (deleted from position 878nt)

pONY3.1

In pONY3 there is an extended 5' untranslated region before the start of the *gagpol* coding sequence. It is likely that this unusually long sequence would compromise expression of the *gagpol* cassette. To improve *gagpol* expression pONY3 is modified to remove the remaining 5' LTR. This is done by cutting pONY3 with *Nar* I and *Eco* RV. The 2.4kb fragment is inserted into pBluescript KS+ (Stratagene) at *Cla* I and *Eco* RV sites to make construct pBSpONY3.0. pBSpONY3.0 is cut with *Xho* I and *Eco* RV. The 2.4kb fragment is inserted into pONY3 at *Xho* I and *Eco* RV to make pONY3.1.

This manipulation removes the 5' LTR up to the *Nar* I site within the primer binding region (386nt). This construct gives a two fold increase in titre and increased protein expression (Figure 10).

pONY3.1 like pONY3 encodes *gag*, *gagpol*, *Tat*, *S2* and *Rev*. Since the *S2* mutation experiments showed that S2 is not required either in the production system or in the EIAV vector genome it is possible to design a *gagpol* expression constructs without S2. Two such constructs, pHORSE and pHORSE3.1, are produced.

pHORSE

pHORSE is made by PCR amplification with EGAGP5'OUTER/EGAGPINNER3 and EGAGP3'OUTER/EGAGPINNER5 using pONY3 as template DNA. The two PCR products are purified pooled and re-amplified using primers EGAGP5'OUTER /EGAGP3'OUTER. This product is inserted into the *Xho* I and *Sal* I sites of pSP72 to make pSP72EIAVgagpolO'lap. pONY 3 is cut with *Pvu* II and *Nco* I and the 4.3kb fragment is inserted into pSP72EIAVgagpolO'lap cut with *Pvu* II and *Nco* I to make pSPEGP. This is cut with *Xho* I and *Sal* I (4.7kb) and inserted into pCI-Neo at the *Xho* I and *Sal* I sites. This construct is called pCIEGP. The RRE is cut out from pEGASUS with *Sal I* (0.7kb) and inserted into pCIEGP construct at the *Sal* I site to make pHORSE.

When this construct is assayed for protein expression in the presence or absence of pCI-Rev (a construct expressing the EIAV Rev open reading frame, see above) it is found to be Rev dependent as expected. However, protein expression is much lower than from pONY3.1. In addition when used in virus production the titre is found to be 100 fold lower than that from pONY3.1.

pHORSE3.1

Unexpectedly when the leader sequence (comprising sequences from the end of U5 of the 5' LTR to the ATG start of *gag* 383 – 524nt) of pONY3.1 is inserted into pHORSE, to make pHORSE3.1, protein expression and virus production improved. pHORSE3.1 is made by replacing the 1.5kb *Xho I/Xba* I of pHORSE with the1.6kb *Xho I/Xba* I of pONY3.1. Titres obtained with pHORSE3.1 are similar to that of pONY3.1. The reason for the slightly lower titre of pHORSE3.1 compared to pONY3.1 may be due the requirement for a four plasmid co-transfection with pHORSE3.1 (due to the Rev dependence of this system). We can conclude therefore that a minimal EIAV vector system should have this leader for maximum *gagpol* expression.

pONY4 and pONY4.1

pONY2.11lacZ contains a deletion in *gag* such that only 373bp of the gag ORF

remains. pONY4 was made by replacing the 5' LTR with the CMV LTR from pEGASUS-1. pEGASUS-1 was cut with *Bgl II/Xho* I releasing a 3.2kb fragment (containing the CMV LTR) which was inserted into pSP72 cut with *Bgl II/Xho I.* This construct was named pSPPEG213. This was cut with *Hpa I/Nar I* and the 1.3kb fragment (encompassing the CMV LTR) was inserted into pONY2.11lacZ cut with *Nae I/Nar I.* pONY4.1 contains a deletion (2.1kb) downstream of the lacZ gene (between the *Sfu I* and *Sal I* sites) such that *tat, S2, env, rev* and RRE, are either missing or severely truncated. pONY4.1 was made by cutting it with *Sfu I/Sal I,* blunt-ended by Klenow polymerase and religated. pONY4G was made by replacing the lacZ gene of pONY4 (*Sac II/Kpn I* and then blunting with Klenow polymerase) with that of GFP from pEGFP-N1 (Clontech) (*Bam HI/Xba I* and then blunting with Klenow polymerase) as a blunt fragment.

Inducible - HRE

pEGHREP450

Cut pegHRElacZ with *Not I* (purify the 3.9kb fragment containing the HRE enhancer/SV40 promoter and the lac Z gene). This fragment is then inserted into pBluescript KS+, (Stratagene), cut with *Not I* to make pBHRElacZ. The orientation is such that lac Z and the Amp gene are in the same orientation. This plasmid can now be used to insert any nucleotide sequence of interest in place of lac Z via the *Nco I/sphI* sites under the control of HRE. This cassette can then be inserted into the EIAV vector genome plasmid pegHRElacZ via the *Not I* sites.

PCR amplify P450 using (Accession number M29874, also known as CYP2B) primers 5' P450 and 3' P450. The target for the PCR could be liver cDNA or a plasmid containing the P450.

5' P450 SEQ ID NO: 59

TTTTCAGACCATGGAACTCAGCGTCC (underlined = *Nco I*)

3' P450. SEQ ID NO: 60

ATCGCATGCTCAGCGGGGCAGGAAGCGGATC (underlined = *Sph I*)

This is P450 PCR fragment is cut with *Nco I* and the 0.3kb fragment is purified and inserted into pBHRElacZ cut with *Nco I*. This gives plasmid pBHREP450del. The PCR fragment is cut with *Aat II/Sph I* and the 1.3kb fragment inserted into plasmid pBHREP450del cut with *Aat II/Sph I* (3.9kb purified). This gives plasmid pBHREP450. This is cut with *Not I* and inserted into pegHRElacZ cut with *Not I* to give pegHREP450.

pONY4HREP450

pBHREP450 is cut with *Not I/sph I* releasing the 1.8kb HRE P450 which can be blunt ended with T4 DNA polymerase and inserted into pONY4.0 cut with *Pst I* and blunt ended to give pONY4HREP450.

pONY4.1HREP450

pBHREP450 is cut with *Not I/sph I* releasing the 1.8kb HRE P450 which can be blunt ended with T4 DNA polymerase and inserted into pONY4.1 cut with *Pst I* and blunt ended to give pONY4.1HREP450.

Constitutive - CMV

pEGASUS4P450

pBHREP450 is cut with *Bsm I/Sph I* to give a 1.5kb fragment which is blunt-ended with T4 DNA polymerase and inserted into pEGASUS4 cut with *Xho I/Spl I* blunt-ended with T4 DNA polymerase to make pEGASUS4P450.

pONY4.0P450

pBHREP450 is cut with *Bsm I/Sph I* to give a 1.5kb fragment which is blunt-ended with T4 DNA polymerase and inserted into pONY4.0 cut with *Xho I* blunt-ended with T4 DNA polymerase to make pONY4.0P450.

pONY4.1P450

pBHREP450 is cut with *Bsm I/Sph I* to give a 1.5kb fragment which is blunt-ended with T4 DNA polymerase and inserted into pONY4.0 cut with *Xho I* blunt-ended with T4 DNA polymerase to make pONY4.1P450.

Production

VSV-G Psedotyped EIAV

The above vector genomes can be used in a three plasmid co-transfection with the EIAV gagpol expression plasmid pONY3.1 and the VSV-G envelope to produce EIAV viral vectors.

Due to the toxicity of VSV-G it is essential to have some form of regulated expression. A temperature sensitive VSV-G cell line has been described in TE671.

In addition a tet inducible system has been described (J Virol 1999 Jan;73(1):576-84. A packaging cell line for lentivirus vectors. Kafri T, van Praag H, Ouyang L, Gage FH, Verma IM.)

Rabies-G Psedotyped EIAV

The above vector genomes can be used in a three plasmid co-transfection with the EIAV gagpol expression plasmid pONY3.1 and the Rabies-G envelope to produce EIAV viral vectors.

Example 15 : The use of monocytes/macrophages or stem cells to deliver the enhanced prodrug activating enzymes.

Peripheral blood mononuclear cells are isolated from human peripheral blood at laboratory scale by standard techniques procedures (Sandlie and Michaelsen 1996 In Antibody engineering: a practical approach. Ed McCafferty *et al.* Chapter 9) and at large scale by elutriation (eg Ceprate from CellPro). Adherent cells (essentially monocytes) are enriched by adherence to plastic overnight and cells can be allowed to differentiate along the macrophage differentiation pathway by culturing adherent cells for 1-3 weeks. Alternatively, one could enrich via CD14 immunomagnetic cell sorting. CD14: LPS and LBP receptor (Wright SD *et al* (1990) Science 249:1431. By this method one can get a separation yield from perpheral blood of 95 - 99% of purity and > 90% recovery with no loss of viability. A variety of transfection methods can be used to introduce vectors into monocytes and macrophages, including particle-mediated DNA delivery (biolistics), electroporation, cationic agent-mediated transfection (eg using Superfect, Qiagen). Each of these methods is carried out according to the manufacturer's instructions, taking into account the parameters to be varied to achieve optimal results as specified by the individual manufacturer. Alternatively, viral vectors may be used such as defective Adenovirus vectors (Microbix Inc or Quantum Biotechnologies Inc) or retroviral vectors such as those described in figure 6.

Stem cells are harvested from peripheral blood after mobilisation with G-CSF and/or cyclophosphamide (Cassel *et al* 1993 Exp Hematol. 21, 585). G-CSF (Amgen) is given at a dose of 10 g/kg/day sub-cutaneously for 7 days. Apheresis and enrichment of stem cells is carried out using the CellPro Stem Cell Separator system (Cassel *et al* op. cit.).

The stem cell enriched population is cultured at 10^5 cells/ml in spent medium from RRV producer cells (Example 1), in the presence f 4 g/ml protamine sulphate and 20 ng/ml IL-3 (Sandoz), 50 ng/ml IL-6 (Sandoz), 100 ng/ml SCF (Amgen) (Santiago-Schwartz *et al* 1992, J. Leuk. Biol. 52, 274; Charbord *et al* 1996, Br. J. Haematol. 94, 449; Dao *et al* 1997, Blood, 89, 446; Piacibello *et al* 1997 Blood 89, 2644). Other cytokines and/or autologous stromal cells prepared as described (Dunbar *et al* 1996 Hum Gene Ther 7, 231) may also be added. After 24h the cells are centrifuged and resuspended in fresh RRV – containing medium with growth factors and protamine sulphate as above. This is repeated after a further 24h and the cells cultured for up to a further 48h. After this time the cells are trypsinised, washed several times in fresh medium by centrifugation and resuspended in Plasma-Lyte A for re-infusion. The total volume for re-infusion is approximately 25 - 50 ml. Patients are infused over a period of up to two hours. The number of cells infused is at least 10^5 cells and may be up to of the order of 10^{12} cells.

Cells may also be matured along the myeloid differentiaition pathway prior to re-infusion according to published methods (Haylock *et al* (1992) Blood 80: 1405-1412).

Monocytes, macrophages or stem cells are transfected with an expression vector capable of expressing enhanced prodrug activating enzymes in human cells. Retroviral vectors including lentiviral vectors described above are suitable. For constitutive high level expression, the enhanced prodrug activating enzyme is expressed in a vector which utilises the hCMV-MIE promoter-enhancer, pCI (Promega). For hypoxia-induced expression, the hCMV promoter is replaced by a promoter containing at least one HRE.

Example 16 : The susceptibility of human tumour cells containing modified prodrug activating enzymes to Cyclophosphamide, Tirapazamine and Mitomycin C.

Human tumour cell lines (Houlbrook *et al* 1994, Oncol (Life Sci.Adv.) 13, 69) are btained from the American Type Culture Collection and grown as monolayers in RPMI 1640 medium supplemented with 2mM glutamine and 10% v/v foetal calf serum.

Plasmids are introduced into the human cancer cell lines, in particular the breast cancer MCF-7, MDA468, T47D and NSCLC cell lines using the technique of electroporation as described in Patterson *et al* ibid. Briefly, cells in exponential growth are harvested with a cell scraper, washed and resuspended in 'cytomix' buffer (Van den Hoff *et al* 1992, Nucl. Acid Res. 20, 2902. 5 x 10^6 cells are mixed with 10ug of linearised plasmid) and subjected to electroporation at 4°C, 960μF, 280V, BioRad. Cells are plated at low density and selected for survival in the antibiotic G418. Individual G418 resistant colonies are picked and expanded to produce independent clones. The clones are analysed for the intracellular distribution of the P450R proteins and for the enzyme activity as described below and by Patterson *et al*. Alternatively genes are delivered by retroviral transfection of cells as described by Soneoka *et al* (op. cit.)

To assay P450 reductase, cell lysates are first prepared by liquid nitrogen freezing and thawing cells in buffer comprising 10mM HEPES (pH7.4), 1mM EDTA, 0.5mM benzamide, 0.5mM PMSF, 1ug/ml trypsin inhibitor. Debris is removed by centrifugation at 1600g at 4°C. The resulting supernatent is split into two aliquots. One aliquot stored as a whole cell lysate after adding glycerol to 10%. The other aliquot is centrifuged at 105,000g for 45min at 2°C. The resulting membrane pellet is dried and resuspended in TRIS buffered saline (pH 7.4)

The P450 reductase activity is determined spectrophotometrically by measuring the NADPH dependent reduction of cytochrome c. Reactions comprise, 400μl of cytochrome c (50uM final concentration), 100ul of 10mM KCN (final concentration 1mM) and 10 to 300 ug of protein lysate, which is either the whole cell lysate or the membrane fraction (10-100ul volume). The reaction is made up to 100μl with 100mM phosphate buffer pH7.6. The reaction is equilibrated to 37°C and initiated by the

addition of 20ul of 10mM NADPH (final concentration 200ul). The rate of reduction of cytochrome c is monitored at 550nm for 3min against a blank without NADPH. Initial rates of reaction are calculated and expressed as nmol cytochrome c reduced per min per mg of lysate protein assuming an extinction coefficient of $21mM^{-1}$. The concentration of P450 is determined from the CO (carbon monoxide) binding spectra (Omura and Sato, 1964, J. Biol. Chem. 239, 2370)

To determine the location of enzymes in cells they are analysed by confocal microscopy. For analysis by confocal microscopy the cells are grown on glass coverslips. Cell monolayers are washed and fixed in 1:1 acetone : ethanol, blocked with).1% BSA and then incubated with a rabbit anti-human P450 reductase polyclonal antibody (Smith GCM, Tew DG and Wolf CR 1994 PNAS USA 91 8710 – 8714) at a 1/100 dilution followed by treatment with anti-rabbit IgG FITC conjugated secondary antibody (Becton Dickinson). Cells are examined using a BioRad MRC1000 or MRC1004. The distribution of wild type P450R, alP450R and FN is compared to the distribution of the NLS derivatives.

The sensitivityof cells to prodrugs is analysed as follows;-

i) Tirapazamine IC_{50}

Tirapazamine is synthesised using standard chemistry procedures as described by Seng and Ley, 1972, Angew., Chem., Int. XI. 1009 and Adams *et al* 1984, Br. J. Cancer, 49, 571.

Dose response curves are determined using the MTT proliferation assay. This is based upon the ability of viable cells to convert a soluble tetrazolium salt, MTT, into purple formazan crystals (Mossman 1983J. Immunol. Methods. 65, 55). Parallel 96 well plates containing cells seeded at a density of 10^3 per well are incubated for up to 8 days. At daily intervals the plates are assayed for formazan production and cell number is derived from a standard curve of optical density versus cell number generated each day. Values

f IC_{50}, the concentration of drug required to reduce the optical density by 50% compared to the untreated controls, are used as the measure of cellular sensitivity to a given treatment. Cells are exposed to Tirapazamine for 3hrs under hypoxic condition and allowed to grow for 96hrs prior to the MTT assay. Hypoxia is generally maintained as described by Patterson *et al* 1995 (op. it) as modified by Patterson *et al* 1997 (op. cit) where catalyst induced hypoxia is used in conjunction with nitrogen purged plasticware and reagents.

ii) Cyclophosphamide IC_{50}

Cyclophosphamide is purchased from Sigma Chemical Company. To test for drug sensitivity 4 x 10^4 cells are plated per well of a 6 well culture dish. CP is added 20hrs after seeding. Cells are allowed to grow for up to 7 days. The final viable cell number is determined by the MTT assay as above as above or by staining for viable cells by Trypan blue dye exclusion as described by Chen *et al* 1996 op. cit.

iii) Mitomycin C (MMC) IC_{50}

Mitomycin C is obtained from Sigma Chemical Company. To test for drug sensitivity 5 x 10^3 cells are plated into the wells of a 96 well micro titre plate and after 16hrs they are treated for 3hrs with dilutions of MMC. Cell are allowed to grow for seven days and then cell survival is assayed by the MTT assay as above (Bligh *et al* 1990 op. cit.)

Example 17 : The analysis of bystander effects by cocultivation.

Cells that have been engineered to express TTS-P450R fusion proteins are mixed with non-engineered cells (control cells) in various ratios. The cell viability after treatment with prodrugs is assessed and the IC_{50} is compared with that for homogeneous cultures of engineered cells and control cells. A bystander effect is indicated when percentage cell death exceeds the initial percentage of engineered cells in the mixture. To determine whether cell to cell contact is required control cells are seeded in a dish and

engineered cells are introduced in a COSTAR insert. The drug is added and survival of the control cells is indicative of a diffusible factor arising from the engineered cells. Methods for determining the bystander effect for ep have been described by Chen *et al* op cit.

Example 18 : Analysis of the in vivo efficacy of enhanced prodrug activating enzymes.

Xenografts of tumour cell lines that have been engineered to express the various enhanced prodrug activating enzymes or combinations are produced in nude mice. The mice are treated with increasing concentrations of the relevant prodrug and tumour growth and cell survival is compared with a control xenograft on the alternate flank.

Female homozygous (nu^+/nu^+) nude athymic Swiss mice 20-30g are used. Tumour cells, for example MCF-7 or MDA231, in the exponential growth phase (2×10^7 per 0.2ml per injection site) are injected subcutane ously into the flank. Each mouse receives two implants, a control tumour on one flank and a prodrug activating enzyme expressing tumour on the other. In some cases there is additional treatment, for example if MCF-7 cells are used the mice are pre-implanted with a 17-Beta oestradiol pellet 1 day prior to implanting the tumour cells (Chen *et al* 1996 op. cit). Drug treatment is initiated when the tumours are approximately 50 to 100mm^2 in size, usually 5 to 6 weeks post implantation. Drugs are administered at an appropriate dose by intra-peritoneal or intra-tumoral injection usually in two administrations separated by 24hrs.

For some animals, at an appropriate time, usually 4 to 48hrs after drug treatment the animals are sacrificed and the tumours are analysed for the expression and action of the prodrug. Tumours are disrupted by standard procedures including mincing, freeze thawing, sonication and treating with collagenase (500µg/ml) depending upon the tumour cell type. Assays for enzyme activity and cell survival are as described above. Some animals are maintained and tum ur size is measured using external calipers.

Example 19 : Analysis f the effect of the intra-tum ral delivery f vectors expressing enhanced prodrug activating enzymes.

Human tumour xenografts are prepared as described above. Once the xenografts have reach about 50 mm they are injected with a 22 gauge needle with vector preparations. Each tumour is injected at least 5 times to give a distribution of the vector throughout the tumour. The animal is treated with prodrug 48hrs after vector injection and tumours are analysed as described above.

Example 20 : Macrophages expressing prodrug activating enzyme

Primary human macrophages were isolated from peripheral blood by standard methods. They were analysed for the expression of the P450 gene and the P450 reductase gene. Surprisingly they had P450 reductase expression but not P450.

Adenoviral vectors were constructed that contain the CMV promoter (Ad.CMV) or the hypoxia responsive HRE promoter (ad.ObHRE). The gene for human cytochrome P4502B6 was inserted into these vectors. P450 expression could be detected in the engineered macrophages. The macrophages were treated with cyclophosphamide and unlike other cells such as tumour cells that have been similarly engineered they were not killed. However, when the P450 engineered macrophages were added to tumour cells or to three dimensional tumour spheroids these were killed in the presence of cyclophosphamide. If the macrophages had been engineered with a marker protein in this case GFP, then the tumour cells all survived. The effect was most dramatic with the Ad.CMV-P450 presumably becuase of the constitutive expression. However, the Ad.HRE-P450 also gave significant killing indicating that one can impose tumour selectivity on the system.

Example 21: Killing of human tumour spheroids with macrophages engineered to express human P450

The results of this experiment are shown in Figure 10. The top three panels show spheroids that have been treated with engineered macrophages but they have not been treated with cyclophosphamide. They all have a discrete edge and look solid. The bottom panel shows what happens if cyclophosphamide is added. With the Ad.CMV-P450 the macrophages the spheroid is completely destroyed and can not be handled for subsequent analysis. With the Ad.HRE-P450 the spheroid is smaller and more diffuse looking and it is very fragile to handle. With Ad.CMV-GFP the spheroid is normal. It is particularly impressive that the tumour target is totally destroyed by Ad.CMV-P450.

CLAIMS

1. A prodrug activating agent comprising:

a) a localisation domain; and

b) a prodrug activation domain for activating a prodrug in a target cell; and

wherein the localisation domain is not a tumour selective antibody.

2. A prodrug activating agent according to claim 1 wherein the localisation domain comprises an intra- or trans-cellular localisation domain.

3. A prodrug activating agent according to claim 1 wherein the localisation domain comprises a biochemical association domain.

4. A prodrug activating agent according to claim 1 wherein the localisation domain comprises a chemical association domain.

5. A prodrug activating agent according to claim 1 wherein the localisation domain comprises a mitochondrial import domain.

6. A prodrug activating agent according to any preceding claim wherein the prodrug activation domain is a prodrug activating enzyme.

7. A prodrug activating agent according to claim 6 wherein the prodrug activating enzyme is a cytochrome P450 or a cytochrome P450 reductase.

8. A prodrug activating agent according to claim 7 wherein the cytochrome P450 is cytochrome P450 2B6.

9. A prodrug activating agent according to any preceding claim in the form of a fusion protein.

10. A prodrug activating agent according to any one of claims 1 to 8 in the form of a nucleic acid sequence encoding the localisation domain and prodrug activation domain.

11. A prodrug activating agent comprising at least one expressable nucleic acid sequence coding for a cytochrome P450 wherein the or each nucleic acid sequence is operably linked to one or more constitutive expression control regulatory element(s), or one or more inducible expression control regulatory element(s).

12. A prodrug activating agent comprising a modified haematopoeitic stem cell (MHSC) comprising at least one expressable nucleic acid sequence coding for a prodrug activation domain wherein the or each nucleic acid sequence is operably linked to one or more constitutive expression control regulatory element(s), or one or more inducible expression control regulatory element(s).

13. A prodrug activating agent according to claim 12 wherein the MHSC is a macrophage.

14. A prodrug activating agent according to claim 12 or claim 13 wherein the nucleic acid sequence encodes a prodrug activating enzyme.

15. A prodrug activating agent according to claim 14 wherein the the prodrug activating enzyme is a cytochrome P450.

16 A prodrug activating agent according to claim 15 wherein the cytochrome P450 is cytochrome P450 2B6.

17. A prodrug activating agent according to any one of claims 11 to 16 wherein the constitutive expression control element is a cytomegalovirus promoter.

18. A prodrug activating agent according to any preceding claim further comprising a non-cytotoxic bioreductive moiety.

19. A nucleic acid vector comprising the nucleic acid sequence as defined in any one of claims 10 to 17.

20. A viral vector comprising the nucleic acid sequence as defined in any one of claims 10 to 17.

21. A viral vector according to claim 20 wherein the viral vector is a retroviral vector, an adenoviral vector, an adeno-associated, a pox, or pox-associated viral vector.

22. A viral vector according to claim 21 wherein the viral vector is a lentiviral vector.

23. A prodrug activating agent according to any one of claims 1 to 18, a nucleic acid vector according to claim 19 or a viral vector according to any one of claims 20 to 22 for use in medicine.

24. A pharmaceutical composition comprising a prodrug activating agent according to any one of claims 1 to 18, a nucleic acid vector according to claim 19 or a viral vector according to any one of claims 20 to 22, optionally admixed with a pharmaceutically acceptable diluent, excipient or carrier.

25. Use of a prodrug activating agent according to any one of claims 1 to 18, a nucleic acid vector according to claim 19 or a viral vector according to any one of claims 20 to 22 in the manufacture of a medicament in the treatment of a tumour, inflammation, atherosclerotic blood vessels and dystrophic muscle fibre termini.

26. A cell transduced with the prodrug activating agent according to any one of claims 1 to 18, a nucleic acid vector according to claim 19 or a viral vector according to any one of claims 20 to 22.

27. A delivery system for the agent of any one of claims 1 to 11 wherein the delivery system comprises one or more retrovirus(es) non-viral expression vector(s), adenovirus(es) or plasmids(s).

28. A method of activating a prodrug comprising contacting a target cell with a prodrug under conditions to allow uptake by the target cell such that on localisation of the prodrug activating agent of any one of claims 1 to 18 the prodrug is activated.

29. A method of producing a viral strain which method comprises introducing a nucleic acid sequence as defined in claims 10 to 16 into the genome of a virus.

30. A method according to claim 31 which comprises introducing the nucleic acid sequence into the genome of the virus by homolgous recombination between the genome and a vector as defined in claim 19.

31. A method of producing a MHSC according to any one of claims 12 to 16 comprising introducing a viral vector according to any one of claims 20 to 22 into a haematopoeitic stem cell.

FIG.1A

SEQ ID NO: 1

```
   1 GGAGACTCCC ACGTGGACAC CAGCTCCACC GTGTCCGAGG CGGTGGCCGA
  51 AGAAGTATCT CTTTTCAGCA TGACGGACAT GATTCTGTTT TCGCTCATCG
 101 TGGGTCTCCT AACCTACTGG TTCCTCTTCA GAAAGAAAAA AGAAGAAGTC
 151 CCCGAGTTCA CCAAAATTCA GACATTGACC TCCTCTGTCA GAGAGAGCAG
 201 CTTTGTGGAA AAGATGAAGA AAACGGGGAG GAACATCATC GTGTTCTACG
 251 GCTCCCAGAC GGGGACTGCA GAGGAGTTTG CCAACCGCCT GTCCAAGGAC
 301 GCCCACCGCT ACGGGATGCG AGGCATGTCA GCGGACCCTG AGGAGTATGA
 351 CCTGGCCGAC CTGAGCAGCC TGCCAGAGAT CGACAACGCC CTGGTGGTTT
 401 TCTGCATGGC CACCTACGGT GAGGGAGACC CCACCGACAA TGCCCAGGAC
 451 TTCTACGACT GGCTGCAGGA GACAGACGTG GATCTCTCTG GGGTCAAGTT
 501 CGCGGTGTTT GGTCTTGGGA ACAAGACCTA CGAGCACTTC AATGCCATGG
 551 GCAAGTACGT GGACAAGCGG CTGGAGCAGC TCGGCGCCCA GCGCATCTTT
 601 GAGCTGGGGT TGGGCGACGA CGATGGGAAC TTGGAGGAGG ACTTCATCAC
 651 CTGGCGAGAG CAGTTCTGGC CGGCCGTGTG TGAACACTTT GGGGTGGAAG
 701 CCACGTTCGA GGAGTCCAGC ATTCGCCAGT ACGAGCTTGT GGTCCACACC
 751 GACATAGATG CGGCCAAGGT GTACATGGGG GAGATGGGCC GGCTGAAGAG
 801 CTACGAGAAC CAGAAGCCCC CCTTTGATGC CAAGAATCCG TTCCTGGCTG
 851 CAGTCACCAC CAACCGGAAG CTGAACCAGG GAACCGAGCG CCACCTCATG
 901 CACCTGGAAT TGGACATCTC GGACTCCAAA ATCAGGTATG AATCTGGGGA
 951 CCACGTGGCT GTGTACCCAG CCAACGACTC TGCTCTCGTC AACCAGCTGG
1001 GCAAAATCCT GGGTGCCGAC CTGGACGTCG TCATGTCCCT GAACAACCTG
1051 GATGAGGAGT CCAACAAGAA GCACCCATTC CCGTGCCCTA CGTCCTACCG
1101 CACGGCCCTC ACCTACTACC TGGACATCAC CAACCCGCCG CGTACCAACG
1151 TGCTGTACGA GCTGGCGCAG TACGCCTCGG AGCCCTCGGA GCAGGAGCTG
1201 CTGCGCAAGA TGGCCTCCTC CTCCGGCGAG GGCAAGGAGC TGTACCTGAG
1251 CTGGGTGGTG GAGGCCCGGA GGCACATCCT GGCCATCCTG CAGGACTGCC
1301 CGTCCCTGCG GCCCCCCATC GACCACCTGT GTGAGCTGCT GCCGCGCCTG
1351 CAGGCCCGCT ACTACTCCAT CGCCTCATCC TCCAAGGTCC ACCCCAACTC
1401 TGTGCACATC TGTGCGGTGG TTGTGGAGTA CGAGACCAAG GCCGGCCGCA
1451 TCAACAAGGG CGTGGCCACC AACTGGCTGC GGGCCAAGGA GCCTGTCGGG
1501 GAGAACGGCG GCCGTGCGCT GGTGCCCATG TTCGTGCGCA AGTCCCAGTT
1551 ACGCCTGCCC TTCAAGGCCA CCACGCCTGT CATCATGGTG GGCCCCGGCA
1601 CCGGGTGGCA CCCTTTCATA GGCTTCATCC AGGAGCGGGC CTGGCTGCGA
1651 CAGCAGGGCA AGGAGGTGGG GGAGACGCTG CTGTACTACG GCTGCCGCCG
1701 CTCGGATGAG GACTACCTGT ACCGGGAGGA GCTGGCGCAG TTCCACAGGG
1751 ACGGTGCGCT CACCCAGCTC AACGTGGCCT TCTCCCGGGA GCAGTCCCAC
1801 AAGGTCTACG TCCAGCACCT GCTAAAGCAA GACCGAGAGC ACCTGTGGAA
1851 GTTGATCGAA GGCGGTGCCC ACATCTACGT CTGTGGGGAT GCACGGAACA
1901 TGGCCAGGGA TGTGCAGAAC ACCTTCTACG ACATCGTGGC TGAGCTCGGG
1951 GCCATGGAGC ACGCGCAGGC GGTGGACTAC ATCAAGAAAG TGATGACCAA
2001 GGGCCGCTAC TCCCTGGACG TGTGGAGCTA GGGGCCTGCC TGCCCCACCC
2051 ACCCCACAGA CTCCGGCCTG TAATCAGCTC TCCTGGCTCC CTCCCGTAGT
2101 CTCCTGGGTG TGTTTGGCTT GGCCTTGGCA TGGGCGCAGG CCCAGTGACA
2151 AAGACTCCTC TGGGCCTGGG GTGCATCCTG CTCAGCCCCC AGGCCAGGTG
2201 AGGTCCACCG GCCCCTGGCA GCACAGCCCA GGGCCTGCAT GGGGGCACCG
2251 GGCTCCATGC CTCTGGAGCC TCTGGCCCTC GGTGGCTGCA CAGAAGGGCT
2301 CTTTCTCTCT GCTGAGCTGG CCCAGCCCCT CCACGTGATT TCCAGTGAGT
2351 GTAAATAATT TTAAATAACC TCTGGCCCTT GGAATAAAGT TCTGTTTTCT
2401 GTA
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SEQ ID NO: 2

GDSHVDTSST VSEAVAEEVS LFSMTDMILF SLIVGLLTYW FLFRKKKEEV PEFTKIQTLT
SSVRESSFVE KMKKTGRNII VFYGSQTGTA EEFANRLSKD AHRYGMRGMS ADPEEYDLAD
LSSLPEIDNA LVVFCMATYG EGDPTDNAQD FYDWLQETDV DLSGVKFAVF GLGNKTYEHF
NAMGKYVDKR LEQLGAQRIF ELGLGDDDGN LEEDFITWRE QFWPAVCEHF GVEATGEESS
IRQYELVVHT DIDAAKVYMG EMGRLKSYEN QKPPFDAKNP FLAAVTTNRK LNQGTERHLM
HLELDISDSK IRYESGDHVA VYPANDSALV NQLGKILGAD LDVVMSLNNL DEESNKKHPF
PCPTSYRTAL TYYLDITNPP RTNVLYELAQ YASEPSEQEL LRKMASSSGE GKELYLSWVV
EARRHILAIL QDCPSLRPPI DHLCELLPRL QARYYSIASS SKVHPNSVHI CAVVVEYETK
AGRINKGVAT NWLRAKEPVG ENGGRALVPM FVRKSQLRLP FKATTPVIMV GPGTGWHPFI
GFIQERAWLR QQGKEVGETL LYYGCRRSDE DYLYREELAQ FHRDGALTQL NVAFSREQSH
KVYVQHLLKQ DREHLWKLIE GGAHIYVCGD ARNMARDVQN TFYDIVAELG AMEHAQAVDY
IKKLMTKGRY SLDVWS

FIG. 1A CONT'D

FIG. 1B

MEMBRANE ANCHOR DOMAIN

FMN

FAD

NADPH

FIG. 2A

SEQ ID NO: 3

TCCTCTGTCA GAGAGAGCAG CTTTGTGGAA AAGATGAAGA AAACGGGGAG
GAACATCATC GTGTTCTACG GCTCCCAGAC GGGGACTGCA GAGGAGTTTG
CCAACCGCCT GTCCAAGGAC GCCCACCGCT ACGGGATGCG AGGCATGTCA
GCGGACCCTG AGGAGTATGA CCTGGCCGAC CTGAGCAGCC TGCCAGAGAT
CGACAACGCC CTGGTGGTTT TCTGCATGGC CACCTACGGT GAGGGAGACC
CCACCGACAA TGCCCAGGAC TTCTACGACT GGCTGCAGGA GACAGACGTG
GATCTCTCTG GGGTCAAGTT CGCGGTGTTT GGTCTTGGGA ACAAGACCTA
CGAGCACTTC AATGCCATGG GCAAGTACGT GGACAAGCGG CTGGAGCAGC
TCGGCGCCCA GCGCATCTTT GAGCTGGGGT TGGGCGACGA CGATGGGAAC
TTGGAGGAGG ACTTCATCAC CTGGCGAGAG CAGTTCTGGC CGGCCGTGTG
TGAACACTTT GGGGTGGAAG CCACTGGCGA GGAGTCCAGC ATTCGCCAGT
ACGAGCTTGT GGTCCACACC GACATAGATG CGGCCAAGGT GTACATGGGG
GAGATGGGCC GGCTGAAGAG CTACGAGAAC CAGAAGCCCC CCTTTGATGC
CAAGAATCCG TTCCTGGCTG CAGTCACCAC CAACCGGAAG CTGAACCAGG
GAACCGAGCG CCACCTCATG CACCTGGAAT TGGACATCTC GGACTCCAAA
ATCAGGTATG AATCTGGGGA CCACGTGGCT GTGTACCCAG CCAACGACTC
TGCTCTCGTC AACCAGCTGG GCAAAATCCT GGGTGCCGAC CTGGACGTCG
TCATGTCCCT GAACAACCTG GATGAGGAGT CCAACAAGAA GCACCCATTC
CCGTGCCCTA CGTCCTACCG CACGGCCCTC ACCTACTACC TGGACATCAC
CAACCCGCCG CGTACCAACG TGCTGTACGA GCTGGCGCAG TACGCCTCGG
AGCCCTCGGA GCAGGAGCTG CTGCGCAAGA TGGCCTCCTC CTCCGGCGAG
GGCAAGGAGC TGTACCTGAG CTGGGTGGTG GAGGCCCGGA GGCACATCCT
GGCCATCCTG CAGGACTGCC CGTCCCTGCG GCCCCCCATC GACCACCTGT
GTGAGCTGCT GCCGCGCCTG CAGGCCCGCT ACTACTCCAT CGCCTCATCC
TCCAAGGTCC ACCCCAACTC TGTGCACATC TGTGCGGTGG TTGTGGAGTA
CGAGACCAAG GCCGGCCGCA TCAACAAGGG CGTGGCCACC AACTGGCTGC
GGGCCAAGGA GCCTGTCGGG GAGAACGGCG GCCGTGCGCT GGTGCCCATG
TTCGTGCGCA AGTCCCAGTT ACGCCTGCCC TTCAAGGCCA CCACGCCTGT
CATCATGGTG GGCCCCGGCA CCGGGTGGCA CCCTTTCATA GGCTTCATCC
AGGAGCGGGC CTGGCTGCGA CAGCAGGGCA AGGAGGTGGG GGAGACGCTG
CTGTACTACG GCTGCCGCCG CTCGGATGAG GACTACCTGT ACCGGGAGGA
GCTGGCGCAG TTCCACAGGG ACGGTGCGCT CACCCAGCTC AACGTGGCCT
TCTCCCGGGA GCAGTCCCAC AAGGTCTACG TCCAGCACCT GCTAAAGCAA
GACCGAGAGC ACCTGTGGAA GTTGATCGAA GGCGGTGCCC ACATCTACGT
CTGTCCCCAT GCACGGAACA TGGCCAGGGA TGTGCAGAAC ACCTTCTACG
ACATCGTGGC TGAGCTCGGG GCCATGGAGC ACGCGCAGGC GGTGGACTAC
ATCAAGAAAC TGATGACCAA GGGCCGCTAC TCCCTGGACG TGTGGAGCTA
G

SEQ ID NO: 4

SSVRESSFVE KMKKTGRNII VFYGSQTGTA EEFANRLSKD AHRYGMRGMS ADPEEYDLAD
LSSLPEIDNA LVVFCMATYG EGDPTDNAQD FYDWLQETDV DLSGVKFAVF GLGNKTYEHF
NAMGKYVDKR LEQLGAQRIF ELGLGDDDGN LEEDFITWRE QFWPAVCEHF GVEATGEESS
IRQYELVVHT DIDAAKVYMG EMGRLKSYEN QKPPFDAKNP FLAAVTTNRK LNQGTERHLM
HLELDISDSK IRYESGDHVA VYPANDSALV NQLGKILGAD LDVVMSLNNL DEESNKKHPF
PCPTSYRTAL TYYLDITNPP RTNVLYELAQ YASEPSEQEL LRKMASSSGE GKELYLSWVV
EARRHILAIL QDCPSLRPPI DHLCELLPRL QARYYSIASS SKVHPNSVHI CAVVVEYETK
AGRINKGVAT NWLRAKEPVG ENGGRALVPM FVRKSQLRLP FKATTPVIMV GPGTGWHPFI
GFIQERAWLR QQGKEVGETL LYYGCRRSDE DYLYREELAQ FHRDGALTQL NVAFSREQSH
KVYVQHLLKQ DREHLWKLIE GGAHIYVCGD ARNMARDVQN TFYDIVAELG AMEHAQAVDY
IKKLMTKGRY SLDVWS

FIG. 2B

SEQ ID NO: 5

CGCCAGTACG AGCTTGTGGT CCACACCGAC ATAGATGCGG CCAAGGTGTA
CATGGGGGAG GAATCCGTTC CTGGCTGCAG TCACCACCAA CCGGAAGCTG
TTGATGCCAA GAATCCGTTC CTGGCTGCAG TCACCACCAA CCGGAAGCTG
AACCAGGGAA CCGAGCGCCA CCTCATGCAC CTGGAATTGG ACATCTCGGA
CTCCAAAATC AGGTATGAAT CTGGGGACCA CGTGGCTGTG TACCCAGCCA
ACGACTCTGC TCTCGTCAAC CAGCTGGGCA AAATCCTGGG TGCCGACCTG
GACGTCGTCA TGTCCCTGAA CAACCTGGAT GAGGAGTCCA ACAAGAAGCA
CCCATTCCCG TGCCCTACGT CCTACCGCAC GGCCCTCACC TACTACCTGG
ACATCACCAA CCCGCCGCGT ACCAACGTGC TGTACGAGCT GGCGCAGTAC
GCCTCGGAGC CCTCGGAGCA GGAGCTGCTG CGCAAGATGG CCTCCTCCTC
CGGCGAGGGC AAGGAGCTGT ACCTGAGCTG GGTGGTGGAG GCCCGGAGGC
ACATCCTGGC CATCCTGCAG GACTGCCCGT CCCTGCGGCC CCCCATCGAC
CACCTGTGTG AGCTGCTGCC GCGCCTGCAG GCCCGCTACT ACTCCATCGC
CTCATCCTCC AAGGTCCACC CCAACTCTGT GCACATCTGT GCGGTGGTTG
TGGAGTACGA GACCAAGGCC GGCCGCATCA ACAAGGGCGT GGCCACCAAC
TGGCTGCGGG CCAAGGAGCC TGTCGGGGAG AACGGCGGCC GTGCGCTGGT
GCCCATGTTC GTGCGCAAGT CCCAGTTACG CCTGCCCTTC AAGGCCACCA
CGCCTGTCAT CATGGTGGGC CCCGGCACCG GGTGGCACCC TTTCATAGGC
GACGCTGCTG TACTACGGCT GCCGCCGCTC GGATGAGGAC TACCTGTACC
GGGAGGAGCT GGCGCAGTTC CACAGGGACG GTGCGCTCAC CCAGCTCAAC
GTGGCCTTCT CCCGGGAGCA GTCCCACAAG GTCTACGTGG AGCACCTGCT
AAAGCAAGAC CGAGAGCACC TGTGGAAGTT GATCGAAGGC GGTGCCCACA
TCTACGTCTG TGGGGATGCA CGGAACATGG CCAGGGATGT GCAGAACACC
TTCTACGACA TCGTGGCTGA GCTCGGGGCC ATGGAGCACG CGCAGGCGGT
GGACTACATC AAGAAACTGA TGACCAAGGG CCGCTACTCC CTGGACGTGT
GGAGCTAG

SEQ ID NO: 6

RQYELVVHTD IDAAKVYMGE MGRLKSYENQ KPPFDAKNPF LAAVTTNRKL NQGTERHLMH
LELDISDSKI RYESGDHVAV YPANDSALVN QLGKILGADL DVVMSLNNLD EESNKKHPFP
CPTSYRTALT YYLDITNPPR TNVLYELAQY ASEPSEQELL RKMASSSGEG KELYLSWVVE
ARRHILAILQ DCPSLRPPID HLCELLPRLQ ARYYSIASSS KVHPNSVHIC AVVVEYETKA
GRINKGVATN WLRAKEPVGE NGGRALVPMF VRKSQLRLPF KATTPVIMVG PGTGWHPFIG
FIQERAWLRQ QGKEVGETLL YYGCRRSDED YLYREELAQF HRDGALTQLN VAFSREQSHK
VYVQHLLKQD REHLWKLIEG GAHIYVCGDA RNMARDVQNT FYDIVAELGA MEHAQAVDYI
KKLMTKGRYS LDVWS

FIG. 3A

SEQ ID NO: 10

CCA AAA AAG AAG AGA AAG GTA

SEQ ID NO: 11

PKKKRKV

FIG. 3B

SEQ ID NO: 19

ATGGCAGCCG GGAGCATCAC CACGCTGCCC GCCTTGCCCG AGGATGGCGG
CAGCGGCGCC TTCCCGCCCG GCCACTTCAA GGACCCCAAG CGGCTGTACT
GCAAAAACGG GGGCTTCTTC CTGCGCATCC ACCCCGACGG CCGAGTTGAC
GGGGTCCGGG AGAAGAGCGA CCCTCACATC AAGCTACAAC TTCAAGCAGA
AGAGAGAGGA GTTGTGTCTA TCAAAGGAGT GTGTGCTAAC CGTTACCTGG
CTATGAAGGA AGATGGAAGA TTACTGGCTT CTAAATGTGT TACGGATGAG
TGTTTCTTTT TTGAACGATT GGAATCTAAT AACTACAATA CTTACCGGTC
AAGGAAATAC ACCAGTTGGT ATGTGGCACT GAAACGAACT GGGCAGTATA
AACTTGGATC CAAAACAGGA CCTGGGCAGA AAGCTATACT TTTTCTTCCA
ATGTCTGCTA AGAGCTGA

SEQ ID NO: 20

MAAGSITTLP ALPEDGGSGA FPPGHFKDPK RLYCKNGGFF LRIHPDGRVD GRVEKSDPHI
KLQLQAEERG VVSIKGVCAN RYLAMKEDGR LLASKCVTDE CFFFERLESN NYNTYRSRKY
TSWYVALKRT GQYKLGSKTG PGQKAILFLP MSAKS

FIG. 3C

SEQ ID NO: 53

CGCAAACGCG GAAGGCAGAC ATACACCCGG TACCAGACTC TAGAGCTAGA GAAGGAGTTT
CACTTCAATC GCTACTTGAC CCGTCGGCGA AGGATCGAGA TCGCCCACGC CCTGTGCCTC
ACGGAGCGCC AGATAAAGAT TTGGTTCCAG AATCGGCGCA TGAAGTGGAA GAAGGAGAAC

SEQ ID NO: 54

RKRERQTYTR YQTLELEKEF HFNRYLTRRR RIEIAHALCL TERQIKIWFQ NRRMKWKKEN

FIG. 3D

SEQ ID NO: 55

ATGACCTCTC GCCGCTCCGT GAAGTCGGGT CCGCGGGAGG TTCCGCGCGA
TGAGTACGAG GATCTGTACT ACACCCCGTC TTCAGGTATG GCGAGTCCCG
ATAGTCCGCC TGACACCTCC CGCCGTGGCG CCCTACAGAC ACGCTCGCGC
CAGAGGGGCG AGGTCCGTTT CGTCCAGTAC GACGAGTCGG ATTATGCCCT
CTACGGGGGC TCGTCATCCG AAGACGACGA ACACCCGGAG GTCCCCCGGA
CGCGGCGTCC CGTTTCCGGG GCGGTTTTGT CCGGCCCGGG GCCTGCGCGG
GCGCCTCCGC CACCCGCTGG GTCCGGAGGG GCCGGACGCA CACCCACCAC
CGCCCCCCGG GCCCCCCGAA CCCAGCGGGT GGCGACTAAG GCCCCCGCGG
CCCCGGCGGC GGAGACCACC CGCGGCAGGA AATCGGCCCA GCCAGAATCC
GCCGCACTCC CAGACGCCCC CGCGTCGACG GCGCCAACCC GATCCAAGAC
ACCCGCGCAG GGGCTGGCCA GAAAGCTGCA CTTTAGCACC GCCCCCCCAA
ACCCCGACGC GCCATGGACC CCCCGGGTGG CCGGCTTTAA CAAGCGCGTC
TTCTGCGCCG CGGTCGGGCG CCTGGCGGCC ATGCATGCCC GGATGGCGGC
GGTCCAGCTC TGGGACATGT CGCGTCCGCG CACAGACGAA GACCTCAACG
AACTCCTTGG CATCACCACC ATCCGCGTGA CGGTCTGCGA GGGCAAAAAC
CGACGCGGCC ACGGCGACTC GAGGGCGTTC TGCGGCGTCG CGCCCCACCG
AGCGACCTCG AGCCCCAGCC CGCTCCGCTT CTCGCCCCAG ACGGCCCGTC
GAGTGA

SEQ ID NO: 56

MTSRRSVKSG PREVPRDEYE DLYYTPSSGM ASPDSPPDTS RRGALQTRSR QRGEVRFVQY
DESDYALYGG SSSEDDEHPE VPRTRRPVSG AVLSGPGPAR APPPPAGSGG AGRTPTTAPR
APRTQRVATK APAAPAAETT RGRKSAQPES AALPDAPAST APTRSKTPAQ GLARKLHFST
APPNPDAPWT PRVAGFNKRV FCAAVGRLAA MHARMAAVQL WDMSRPRTDE DLNELLGITT
IRVTVCEGKN LLQRANELVN PDVVQDVDAA TATRGRSAAS RPTERPRAPA RSASRPRRPV
E

FIG. 3E

SEQ ID NO: 33

GGCAGCCTGG CCGCGCTGAC CGCGCACCAG GCTTGCCACC TGCCGCTGGA
GACTTTCACC CGTCATCGCC AGCCGCGCGG CTGGGAACAA CTGGAGCAGT
GCGGCTCTCC GGTGCAGCGG CTGGTCGCCC TCTACCTGGC GGCGCGGCTG
TCGTGGAACC AGGTCGACCA GGTGATCCGC AACGCCCTGG CCAGCCCCGG
CAGCGGCGGC GACCTGGGCG AAGCGATCCG CGAGCAGCCG GAGCAGGCCC
GTCTGGCCCT GACCCTGGCC GCCGCCGAGA GCGAGCGCTT CGTCCGGCAG
GGCACCGGCA ACGACGAGGC CGGCGCGGCC AAC

SEQ ID NO: 34

GGSLAALTAH QACHLPLETF TRHRQPRGWE QLEQCGYPVQ RLVALYLAAR LSWNQVDQVI
RNALASPGSG GDLGEAIREQ PEQARLALTL AAAESERFVR QGTGNDEAGA AN

FIG. 3F

SEQ ID NO: 25

ccaccatggg atggagctgt atcatcctct tcttggtagc aacagctaca ggtgtccact cc

SEQ ID NO: 26

GAGGTCCAGC TTCAGCAGTC TGGACCTGAC CTGGTGAAGC CTGGGGCTTC
AGTGAAGATA TCCTGCAAGG CTTCTGGTTA CTCATTCACT GGCTACTACA
TGCACTGGGT GAAGCAGAGC CATGGAAAGA GCCTTGAGTG GATTGGACGT
ATTAATCCTA ACAATGGTGT TACTCTCTAC AACCAGAAAT TCAAGGACAA
GGCCATATTA ACTGTAGACA AGTCATCCAC CACAGCCTAC ATGGAGCTCC
GCAGCCTGAC ATCTGAGGAC TCTGCGGTCT ATTACTGTGC AAGATCTACT
ATGATTACGA ACTATGTTAT GGACTACTGG GGTCAAGTAA CCTCAGTCAC
CGTCTCCTCA GGTGGTGGTG GGAGCGGTGG TGGCGGCACT GGCGGCGGCG
GATCTAGTAT TGTGATGACC CAGACTCCCA CATTCCTGCT TGTTTCAGCA
GGAGACAGGG TTACCATAAC CTGCAAGGCC AGTCAGAGTG TGAGTAATGA
TGTAGCTTGG TACCAACAGA AGCCAGGGCA GTCTCCTACA CTGCTCATAT
CCTATACATC CAGTCGCTAC GCTGGAGTCC CTGATCGCTT CATTGGCAGT
GGATATGGGA CGGATTTCAC TTTCACCATC AGCACTTTGC AGGCTGAAGA
CCTGGCAGTT TATTTCTGTC AGCAAGATTA TAATTCTCCT CCGACGTTCG
GTGGAGGCAC CAAGCTGGAA ATCAAACGG

SEQ ID NO: 27

EVQLQQSGPD LVKPGASVKI SCKASGYSFT GYYMHWVKQS HGKSLEWIGR
INPNNGVTLY NQKFKDKAIL TVDKSSTTAY MELRSLTSED SAVYYCARST
MITNYVMDYW GQVTSVTVSS GGGGSGGGGT GGGGSSIVMT QTPTFLLVSA
GDRVTITCKA SQSVSNDVAW YQQKPGQSPT LLISYTSSRY AGVPDRFIGS
GYGTDFTFTI STLQAEDLAV YFCQQDYNSP PTFGGGTKLE IKR

8/29

FIG. 4

SEQ ID NO: 45

```
   1 CAGACCATGG AACTCAGCGT CCTCCTCTTC CTTGCACTCC TCACAGGACT
  51 CTTGCTACTC CTGGTTCAGC GCCACCCTAA CACCCATGAC CGCCTCCCAC
 101 CAGGGCCCCG CCCTCTGCCC CTTTTGGGAA ACCTTCTGCA GATGGATAGA
 151 AGAGGCCTAC TCAAATCCTT TCTGAGGTTC CGAGAGAAAT ATGGGGACGT
 201 CTTCACGGTA CACCTGGGAC CGAGGCCCGT GGTCATGCTG TGTGGAGTAG
 251 AGGCCATACG GGAGGCCCTT GTGGACAAGG CTGAGGCCTT CTCTGGCCGG
 301 GGAAAAATCG CCATGGTCGA CCCATTCTTC CGGGGATATG GTGTGATCTT
 351 TGCCAATGGA AACCGCTGGA AGGTGCTTCG GCGATTCTCT GTGACCACTA
 401 TGAGGGACTT CGGGATGGGA AAGCGGAGTG TGGAGGAGCG GATTCAGGAG
 451 GAGGCTCAGT GTCTGATAGA GGAGCTTCGG AAATCCAAGG GGGCCCTCAT
 501 GGACCCCACC TTCCTCTTCC AGTCCATTAC CGCCAACATG ATCTGCTCCA
 551 TCGTCTTTGG AAAACGATTC CACTACCAAG ATCAAGAGTT CCTGAAGATG
 601 CTGAACTTGT TCTACCAGAC TTTTTCACTC ATCAGCTCTG TATTCGGCCA
 651 GCTGTTTGAG CTCTTCTCTG GCTTCTTGAA ATACTTTCCT GGGGCACACA
 701 GGCAAGTTTA CAAAAACCTG CAGGAAATCA ATGCTTACAT TGGCCACAGT
 751 GTGGAGAAGC ACCGTGAAAC CCTGGACCCC AGCGCCCCCA AGGACCTCAT
 801 CGACACCTAC CTGCTCCACA TGGAAAAAGA GAAATCCAAC GCACACAGTG
 851 AATTCAGCCA CCAGAACCTC AACCTCAACA CGCTCTCGCT CTTCTTTGCT
 901 GGCACTGAGA CCACCAGCAC CACTGTCCGC TACGGCTTCC TGCTCATGCT
 951 CAAATACCCT CATGTTGCAG AGAGAGTCTA CAGGGAGATT GAACAGGTCA
1001 TTGGCCCACA TCGCCCGCCA GAGCTTCATG ACCGAGCCAA AATGCCATAC
1051 ACAGAGGCAG TCATCTATGA GATTCAGAGA TTTTCCGACC TTCTCCCCAT
1101 GGGTGTGCCC CACATTGTCA CCCAACACAC CAGCTTCCGA GGGTACATCA
1151 TCCCCAAGGA CACAGAAGTA TTTCTCATCC TGAGCACTGC TCTCCATGAC
1201 CCACACTACT TTGAAAAACC AGACGCCTTC AATCCTGACC ACTTTCTGGA
1251 TGCCAATGGG GCACTGAAAA AGACTGAAGC TTTTATCCCC TTCTCCTTAG
1301 GGAAGCGGAT TTGTCTTGGT GAAGGCATCG CCCGTGCGGA ATTGTTCCTC
1351 TTCTTCACCA CCATCCTCCA GAACTTCTCC ATGGCCAGCC CCGTGGCCCC
1401 AGAAGACATC GATCTGACAC CCCAGGAGTG TGGTGTGGGC AAAATACCCC
1451 CAACATACCA GATCCGCTTC CTGCCCCGCT GAAGGGGCTG AGGGAAGGGG
1501 GTCAAAGGAT TCCAGGGTCA TTCAGTGTCC CCGCCTCTGT AGACAATGGC
1551 TCTGACTCCC CGCAACTTCC TGCCTCTGAG AGACCTGCTA CAAGCCAGCT
1601 TCCTTCCCCT CCATGGCACC AGTTGTCTGA GGTCACATTG CAAGTGAGTG
1651 CAGGAGTGAG ATTATCGAAA ATTATAATAT ACAAAATCAT ATATATATAT
1701 ATGTTCTTGT TTTTTGAGAC AGAGTCTCAC ACTGTTGCCC AGGCTGGAGT
1751 GCAGTGGCGT GATCTTGGCT CACTGCAACC TCCACCCCCG GGGATCAAGC
1801 AACTCTCCTG CCTCAGCCTC CCTAGAGGCT GGGATTACAG GCATGCACTA
1851 CCACGCTTGG CTAATTTTTG TATTTTTAGT AGAGATGGGG TTTCACTGTG
1901 TAGGCCAGGC TGGTCTCGAA CTCCTGAACT CAAGTGATTC ACCCACCTTA
1951 GCCTCCCAAA GTGCTGGGAT TACAGGCGTG AGTCACCGTG CCCAGCCATG
2001 TATATATATA ATTTTAAAAA TTAAGCTGAA ATTCACATAA CATAAAATTA
2051 GCCGTTTTAA AGTGTAAAAT TTAGTGGCGT GTGGTTCATT CACAAAGCTG
2101 TACAACCACC ACCATCTAGT TCCAAACATT TTCTTTTTTT CTGAGATGGA
2151 GTCTCACTCT GTCACCCAGG TTCGAGTTCA GTGGTGCCAT CTCTGTCCAC
2201 TGCAACCTCC ACATCCTGGG TTCAAGTGAT TCTCCTGCCT CAGCCTCTGG
2251 AGGAGCTGGT ATCACAGGCG TCCCCCACCA CGCCTGGCTA AATTTTGTAT
2301 TTTTAGGTGG TCTTGAACTC CTGATGTCAG GTGATTCTCC TAGCTCCAAA
2351 TGTTTTCATT ATCTCTCCCC CAACAAAACC CATACCTATC AAGCTGTCAC
2401 TCCCCATACC CCATTCTCTT TTTCATCTCG GCCCCTGTCA ATCTGGTTTT
2451 TGTCACTATG GACTTACCAA TTCTGAATAT TTCCCATAAA CAGAATCATA
2501 CAATATTTGA TTTTTTTTTT TTTTTGAAAC TAAGCCTTGC TCTGTCTCCC
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2551 AGGCTGGAGT GCTATGGTGC AATTTTTGTT CACTGCAACC TCTGCCTTCC
2601 AAGATCAAGA GATTCTCCAG TCTCAGCTCC CAAGTAGCTG GGATTACAGG
2651 CATGTACTAC CATGCCTGGC TAATTTTCTT GTAGTTTTAG TAGGGACATG
2701 TTGGCCAGGC TGGTGGTGAG CTCCTGGCCT CAGGTGATCC ACCCACCTGA
2751 GTGTTCCTAA GTGCTGATAT TACAGGCATA ATATGTGATC TTTTGTGTCT
2801 GGTTGCTTTC ATGTTGAATG CTATTTTTGA GGTTCGTGCC TGTTGTAGAC
2851 CACAGTCACA CACTGCTGTA GTCTTCCCGA GTCCTCATTC CCAGCTGCCT
2901 CTTCCTACTG CTTCCGTCTA TCAAAAAGCC CCCTTGGCCC AGGTTCCCTG
2951 AGCTGTGGGA TTCTGCACTG GTGCTTTGGA TTCCCTGATA TGTTCCTTCA
3001 AATCTGCTGA GAATTAAATA AACATCTCTA AGCCTGACC TCCCC
```

SEQ ID NO: 46

```
MELSVLLFLA LLTGLLLLLV QRHPNTHDRL PPGPRPLPLL GNLLQMDRRG LLKSFLRFRE
KYGDVFTVHL GPRPVVMLCG VEAIREALVD KAEAFSGRGK IAMVDPFFRG YGVIFANGNR
WKVLRRFSVT TMRDFGMGKR SVEERIQEEA QCLIEELRKS KGALMDPTFL FQSITANIIC
SIVFGKRFHY QDQEFLKMLN LFYQTFSLIS SVFGQLFELF SGFLKYFPGA HRQVYKNLQE
INAYIGHSVE KHRETLDPSA PKDLIDTYLL HMEKEKSNAH SEFSHQNLNL NTLSLFFAGT
ETTSTTLRYG FLLMLKYPHV AERVYREIEQ VIGPHRPPEL HDRAKMPYTE AVIYEIQRFS
DLLPMGVPHI VTQHTSFRGY IIPKDTEVFL ILSTALHDPH YFEKPDAFNP DHFLDANGAL
PPTYQIRFLP R
```

FIG. 4CONT'D

FIG. 5

SEQ ID NO: 52

```
cgagctcagc aggtttcccc aactgacaca aaacgtgcaa cttgaaactc
cgcctggtct ttggaggtct agaggggtaa cactttgtac tgcgtttggc
tccacgctcg atccactggc gagtgttagt aacagcactg ttgcttcgta
gcggagcatg acggccgtgg gaactcctcc ttggtaacaa ggacccacgg
ggccaaaagc cacgcccaca cgggcccgtc atgtgtgcaa ccccagcacg
gcgactttac tgcgaaaccc actttaaagt gacattgaaa ctggtaccca
cacactggtg acaggctaag gatgcccttc aggtaccccg aggtaacacg
cgacactcgg gatctgagaa ggggactggg gcttctataa aagcgctcgg
tttaaaaagc ttctatgcct gaataggtga ccggaggtcg gcacctttcc
tttgcaatta atgaccct gagctcc
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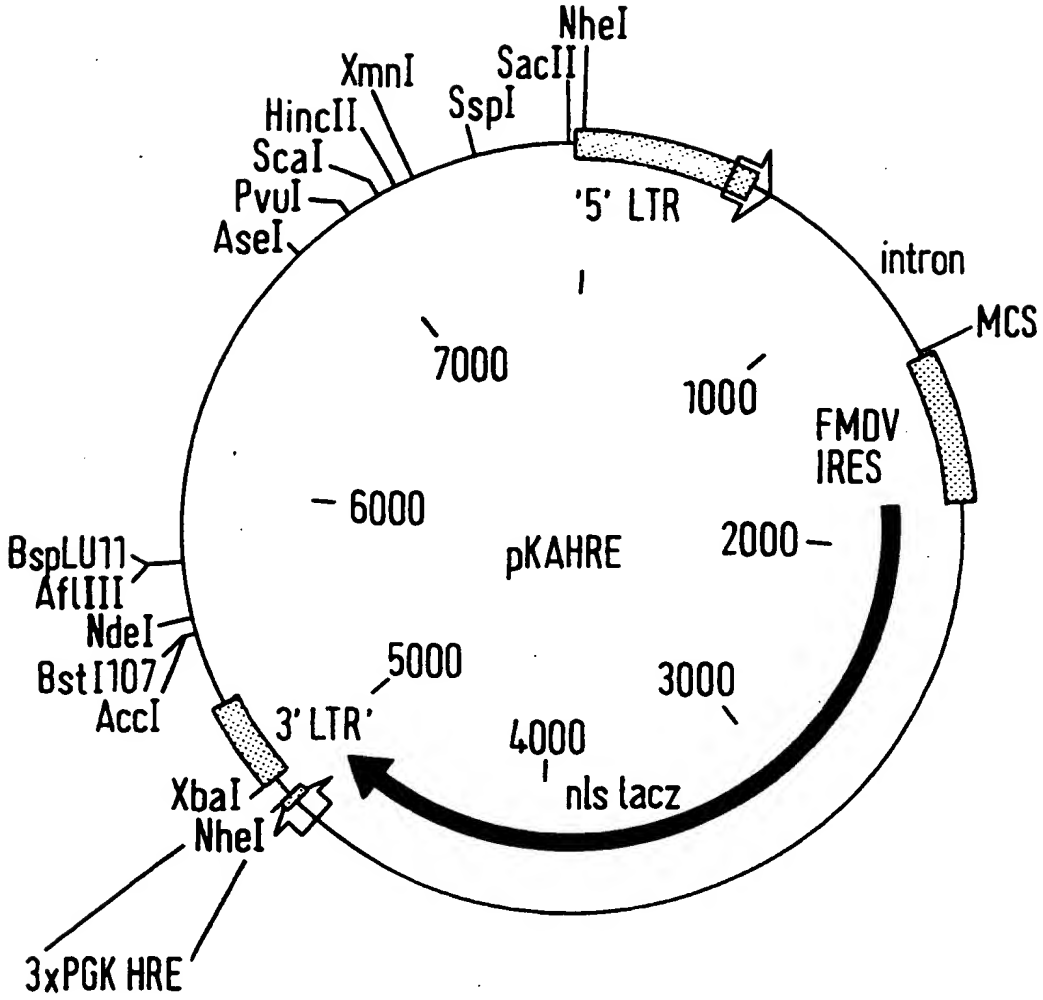

NheI
SacII
XmnI
SspI
HincII
ScaI
PvuI
AseI
'5' LTR
intron
MCS
7000
1000
FMDV
IRES
6000
pKAHRE
2000
BspLU11
AflIII
NdeI
BstI107
AccI
5000
3000
3' LTR'
4000
nls lacz
XbaI
NheI
3xPGK HRE

FIG. 6A

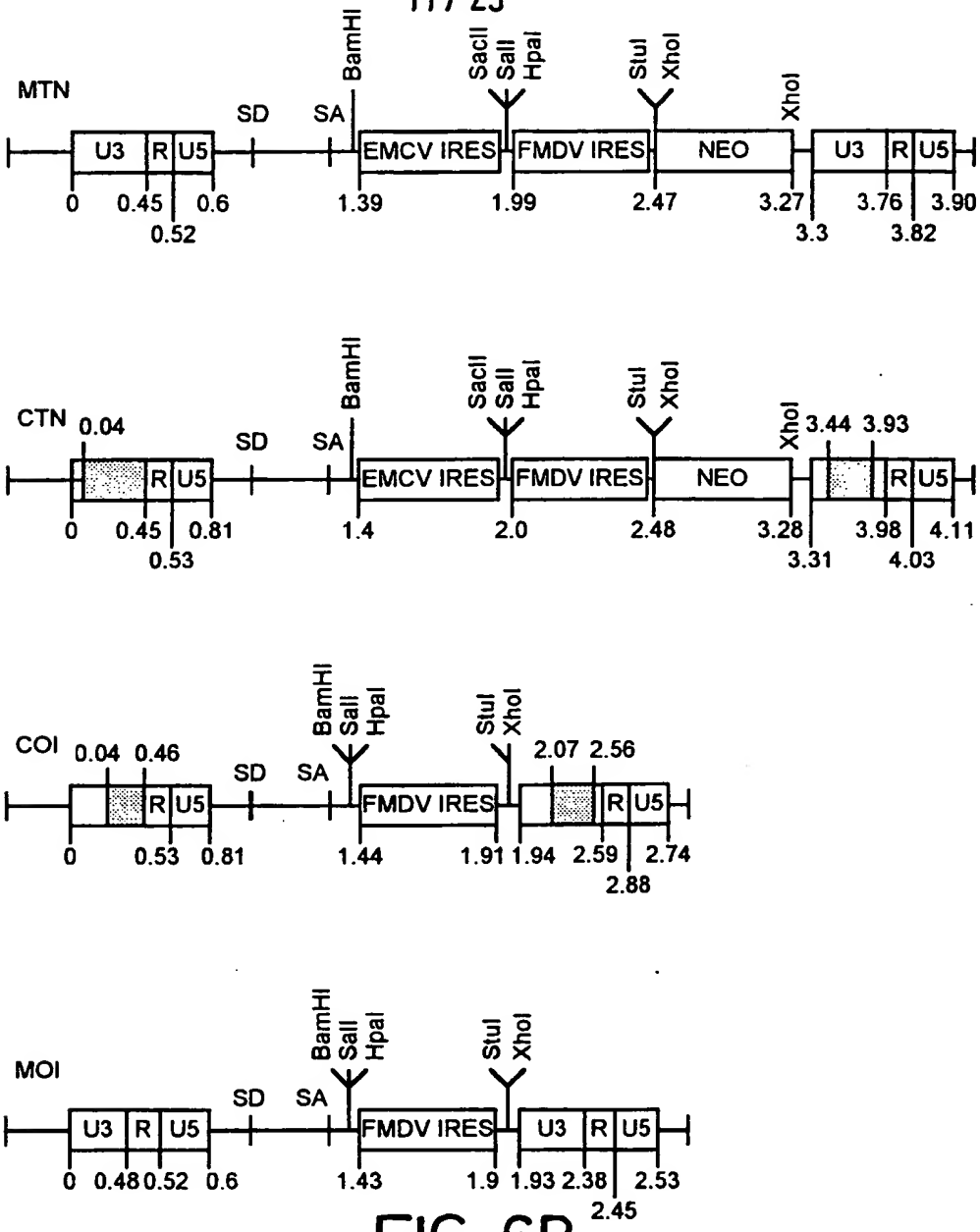

MTN
SD
SA
BamHI
SacII
SalI
HpaI
StuI
XhoI
XhoI
U3
R
U5
EMCV IRES
FMDV IRES
NEO
U3
R
U5
0
0.45
0.52
0.6
1.39
1.99
2.47
3.27
3.3
3.76
3.82
3.90
CTN
0.04
SD
SA
BamHI
SacII
SalI
HpaI
StuI
XhoI
XhoI
3.44
3.93
R
U5
EMCV IRES
FMDV IRES
NEO
R
U5
0
0.45
0.53
0.81
1.4
2.0
2.48
3.28
3.31
3.98
4.03
4.11
COI
0.04
0.46
SD
SA
BamHI
SalI
HpaI
StuI
XhoI
2.07
2.56
R
U5
FMDV IRES
R
U5
0
0.53
0.81
1.44
1.91
1.94
2.59
2.74
2.88
MOI
SD
SA
BamHI
SalI
HpaI
StuI
XhoI
U3
R
U5
FMDV IRES
U3
R
U5
0
0.48
0.52
0.6
1.43
1.9
1.93
2.38
2.53
2.45

FIG. 6B

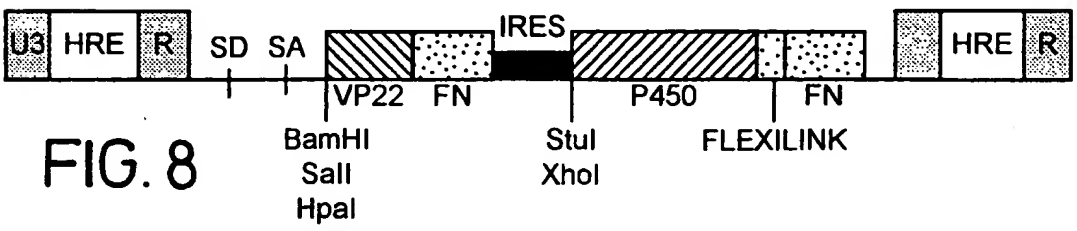

U3
HRE
R
SD
SA
IRES
VP22
FN
P450
FN
HRE
R
BamHI
SalI
HpaI
StuI
XhoI
FLEXILINK

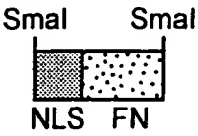
FIG. 8

FIG. 7

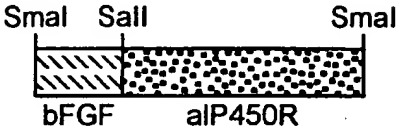
GENE FUSION CONSTRUCTIONS

1. SV40NLS-alP450R

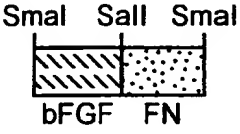
2. SV40NLS-FN



3. bFGF-alP450R

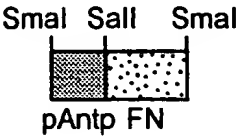


4. bFFG-FN

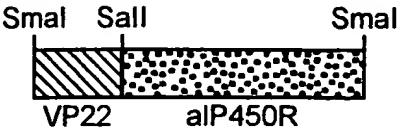


5. Ant-alP450R

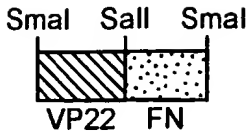
6. Ant-FN



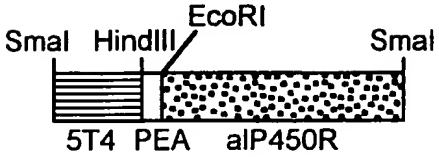
7. VP-alP450R



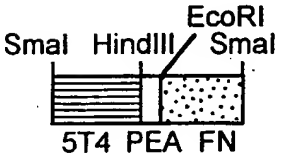
8. VP-FN



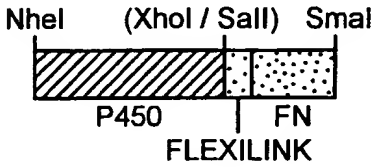
9. 5T4-PEA-aIP450R



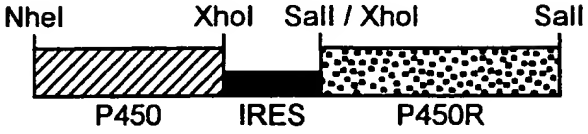
10. 5T4-PEA-FN



11. P450-FN



12. P450-IRES-P450R



13. ST4-aIP450R-MTS

14. ST4-FN-MTS

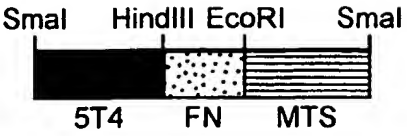


FIG. 7 CONT'D

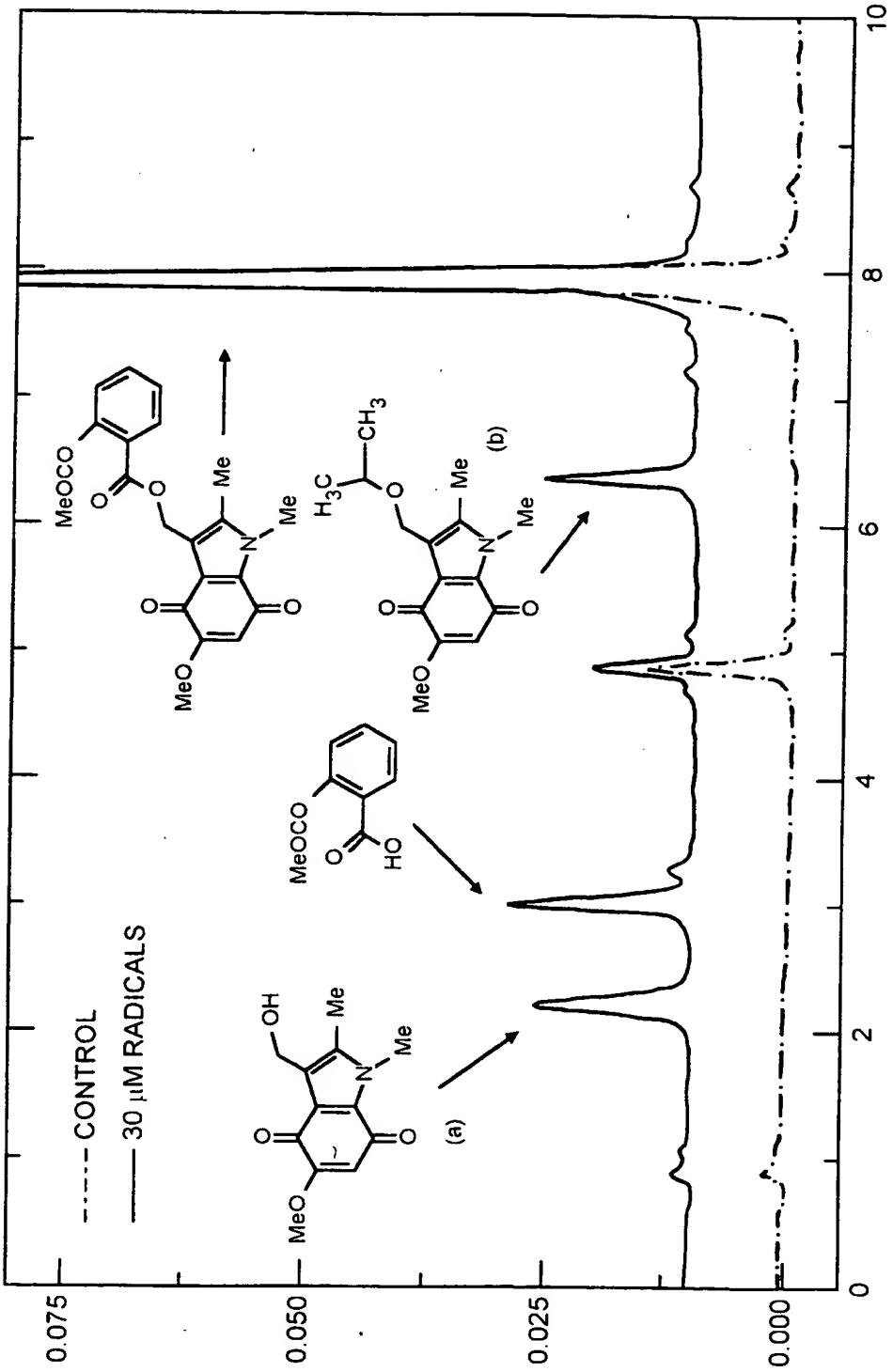

CONTROL
30 μM RADICALS
TIME (MIN)
0.075
0.050
0.025
0.000
0
2
4
6
8
10
(a)
(b)
MeO
MeOCO
HO
OH
Me
N
O
H_3C
CH_3

FIG. 9

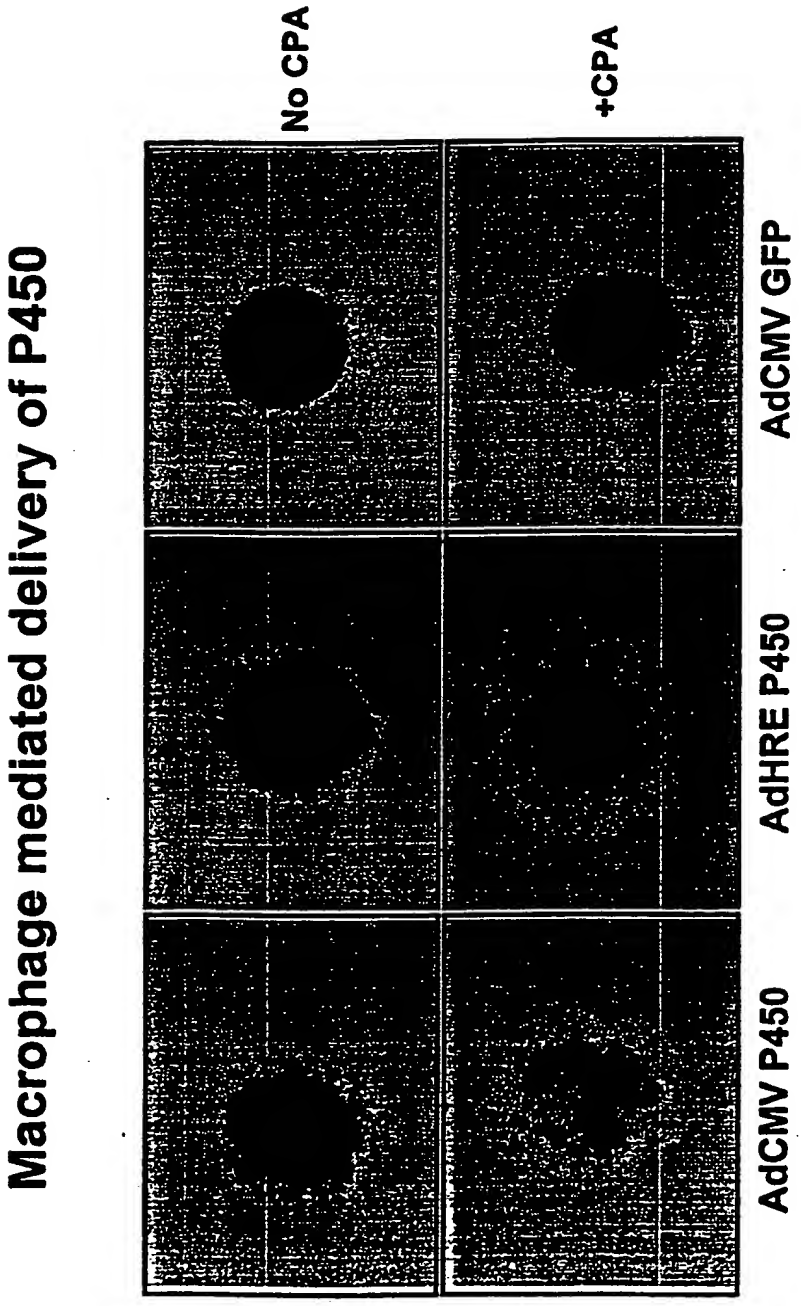

Macrophage mediated delivery of P450
No CPA
+CPA
AdCMV GFP
AdHRE P450
AdCMV P450

FIG. 10

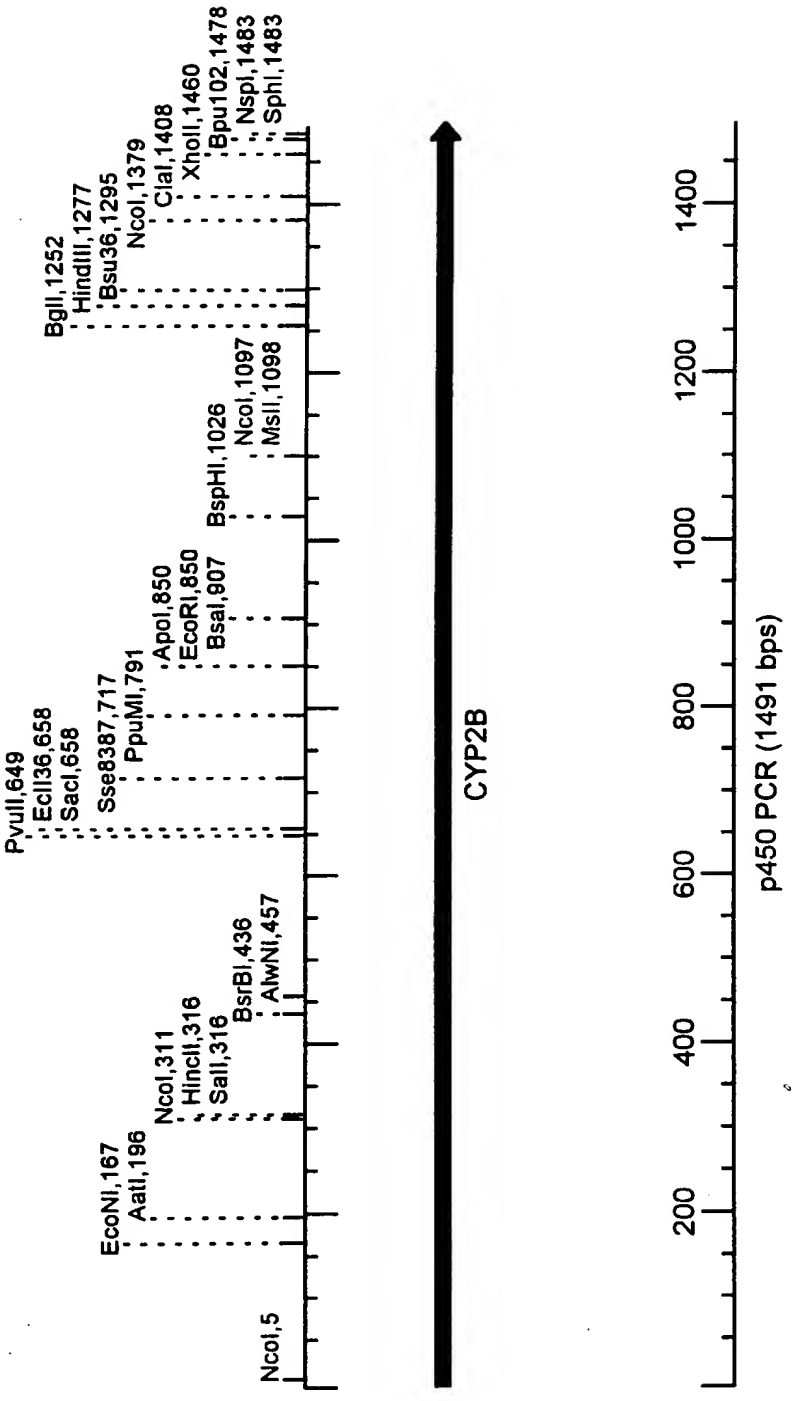

PvuII,649
EclI36,658
SacI,658
Sse8387,717
PpuMI,791
ApoI,850
EcoRI,850
BsaI,907
BspHI,1026
NcoI,1097
MslI,1098
BglI,1252
HindIII,1277
Bsu36,1295
NcoI,1379
ClaI,1408
XhoII,1460
Bpu102,1478
NspI,1483
SphI,1483
EcoNI,167
AatII,196
NcoI,311
HincII,316
SalI,316
BsrBI,436
AlwNI,457
NcoI,5
CYP2B
200
400
600
800
1000
1200
1400
p450 PCR (1491 bps)

FIG. 11

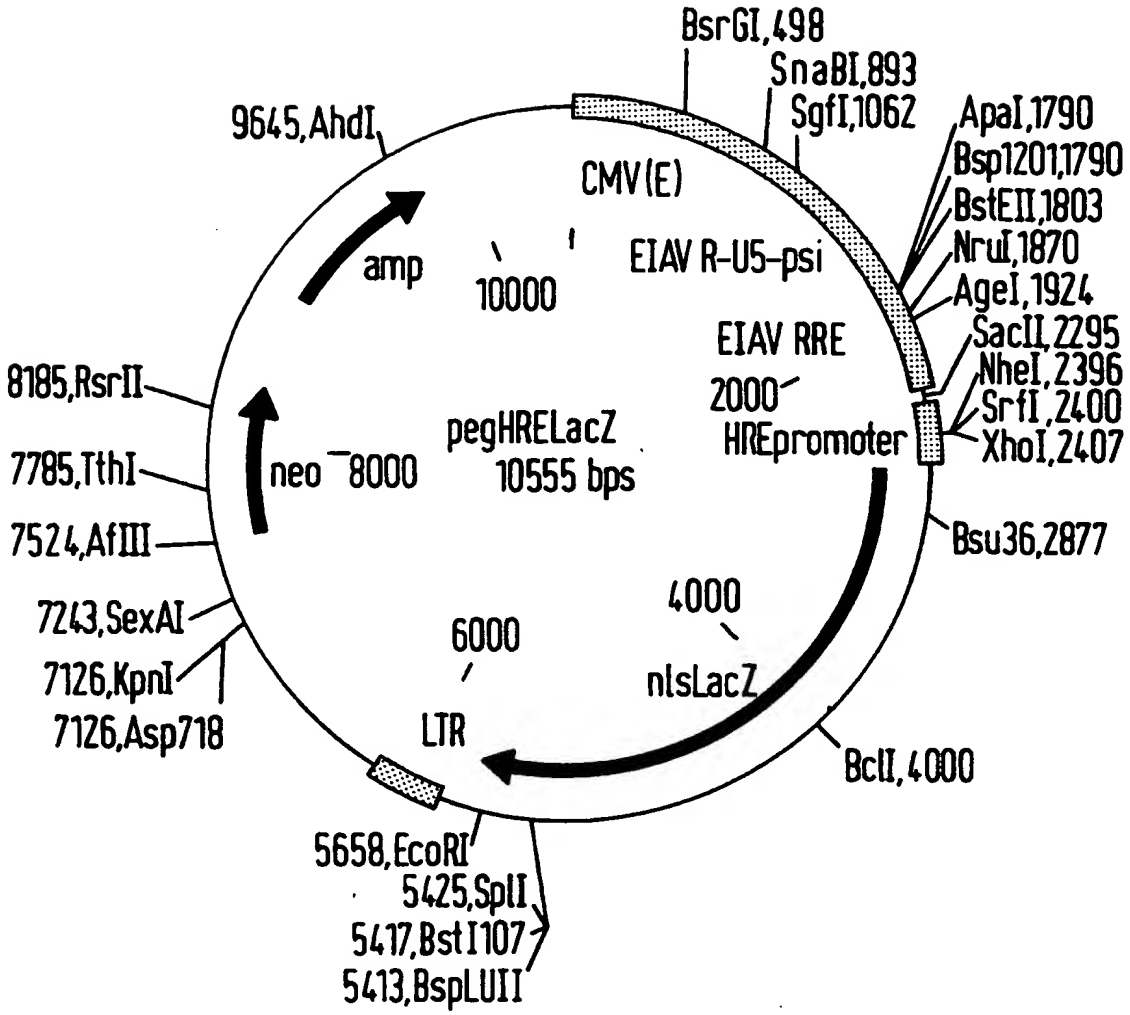

BsrGI,498
SnaBI,893
SgfI,1062
ApaI,1790
Bsp1201,1790
BstEII,1803
NruI,1870
AgeI,1924
SacII,2295
NheI,2396
SrfI,2400
XhoI,2407
Bsu36,2877
BclI,4000
5658,EcoRI
5425,SplI
5417,BstI107
5413,BspLUII
LTR
7126,Asp718
7126,KpnI
7243,SexAI
7524,AflII
7785,TthI
8185,RsrII
9645,AhdI
CMV(E)
amp
10000
EIAV R-U5-psi
EIAV RRE
2000
HREpromoter
pegHRELacZ
10555 bps
neo
8000
4000
nlsLacZ
6000

FIG.12

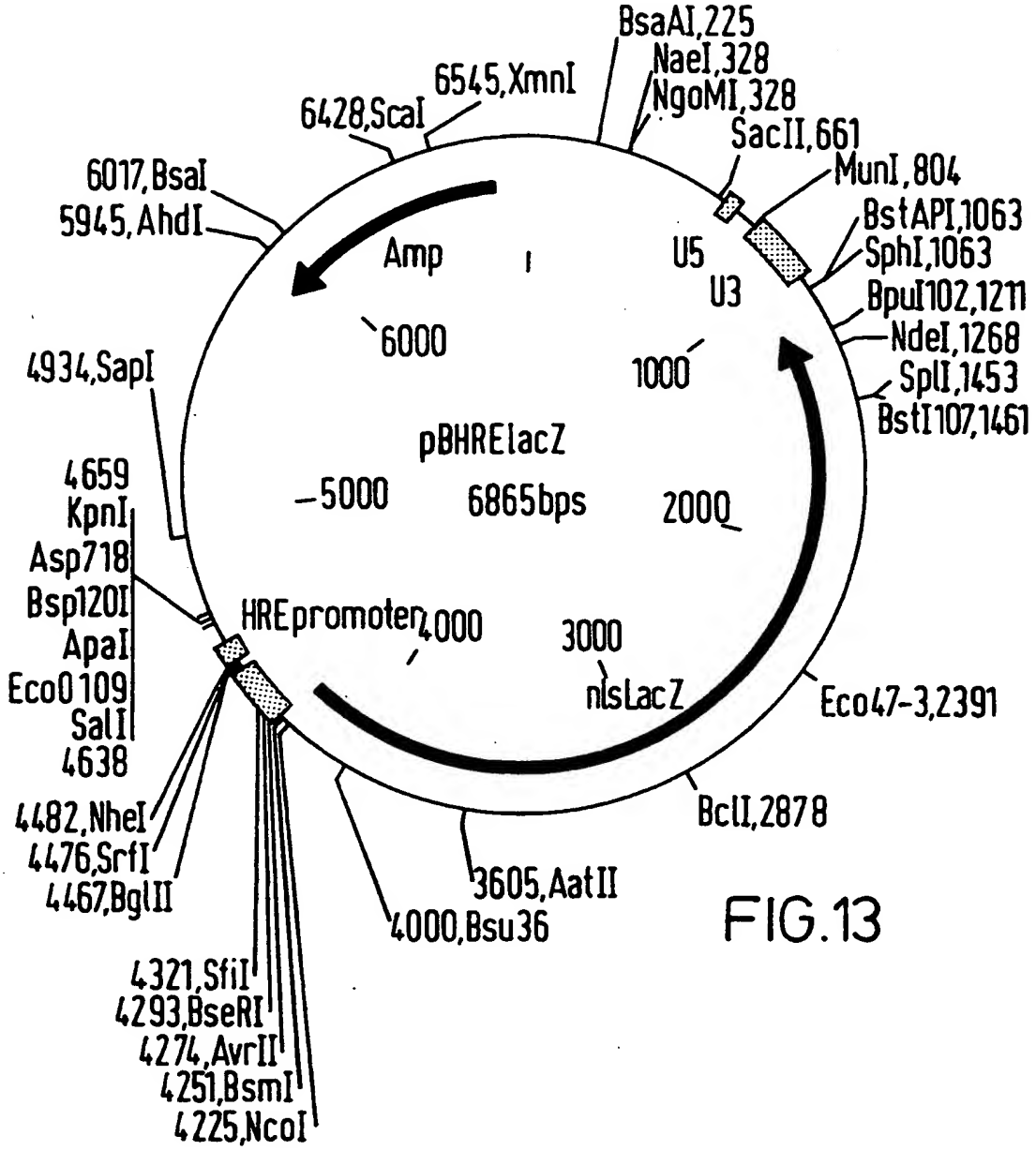

BsaAI,225
NaeI,328
NgoMI,328
SacII,661
MunI,804
BstAPI,1063
SphI,1063
BpuI102,1211
NdeI,1268
SplI,1453
BstI107,1461
Eco47-3,2391
BclI,2878
3605,AatII
4000,Bsu36
4321,SfiI
4293,BseRI
4274,AvrII
4251,BsmI
4225,NcoI
4482,NheI
4476,SrfI
4467,BglII
4659
KpnI
Asp718
Bsp120I
ApaI
Eco0109
SalI
4638
4934,SapI
6017,BsaI
5945,AhdI
6428,ScaI
6545,XmnI
Amp
U5
U3
6000
1000
2000
3000
4000
5000
pBHRElacZ
6865bps
HREpromoter
nlsLacZ

FIG.13

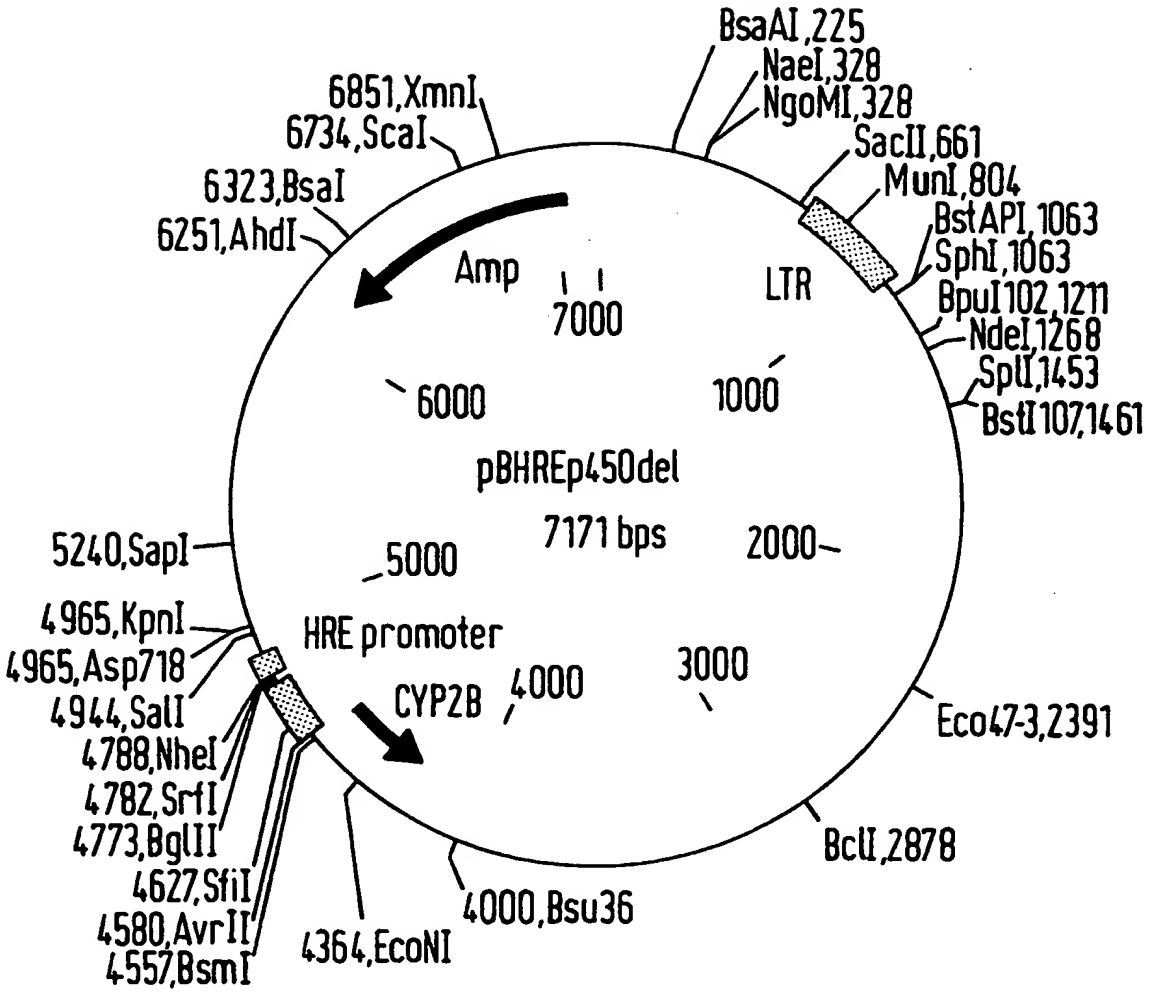

BsaAI,225
NaeI,328
NgoMI,328
SacII,661
MunI,804
BstAPI,1063
SphI,1063
BpuI102,1211
NdeI,1268
SplI,1453
BstI107,1461
Eco47-3,2391
BclI,2878
4000,Bsu36
4364,EcoNI
4557,BsmI
4580,AvrII
4627,SfiI
4773,BglII
4782,SrfI
4788,NheI
4944,SalI
4965,Asp718
4965,KpnI
5240,SapI
6251,AhdI
6323,BsaI
6734,ScaI
6851,XmnI
Amp
LTR
HRE promoter
CYP2B
pBHREp450del
7171 bps
1000
2000
3000
4000
5000
6000
7000

FIG.14

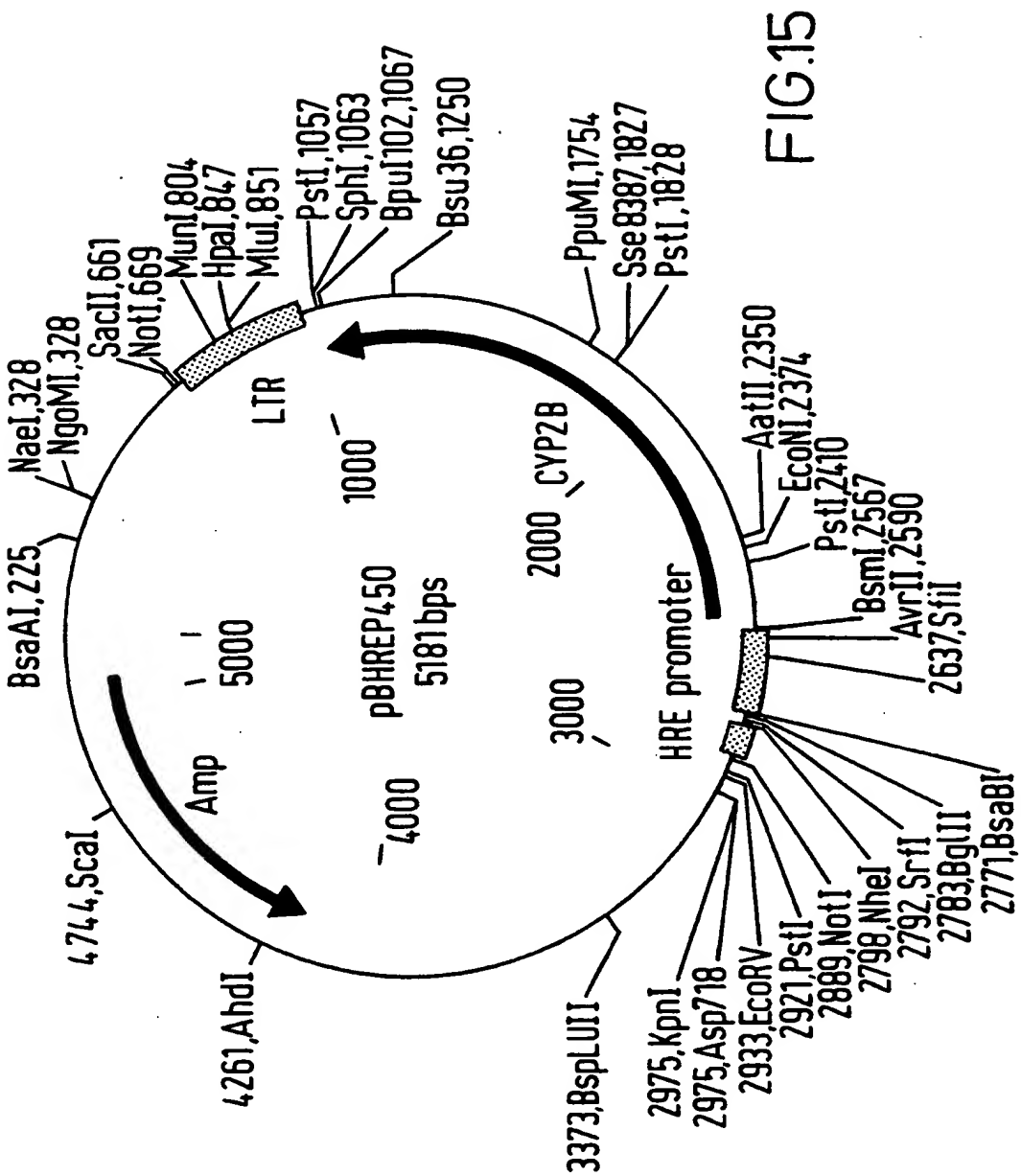

pBHREP450
5181bps
1000
2000
3000
4000
5000
LTR
CYP2B
HRE promoter
Amp
BsaAI,225
NaeI,328
NgoMI,328
SacII,661
NotI,669
MunI,804
HpaI,847
MluI,851
PstI,1057
SphI,1063
Bpu1102,1067
Bsu36,1250
PpuMI,1754
Sse8387,1827
PstI,1828
AatII,2350
EcoNI,2374
PstI,2410
BsmI,2567
AvrII,2590
2637,SfiI
2771,BsaBI
2783,BglII
2792,SrfI
2798,NheI
2889,NotI
2921,PstI
2933,EcoRV
2975,Asp718
2975,KpnI
3373,BspLUII
4261,AhdI
4744,ScaI

FIG.15

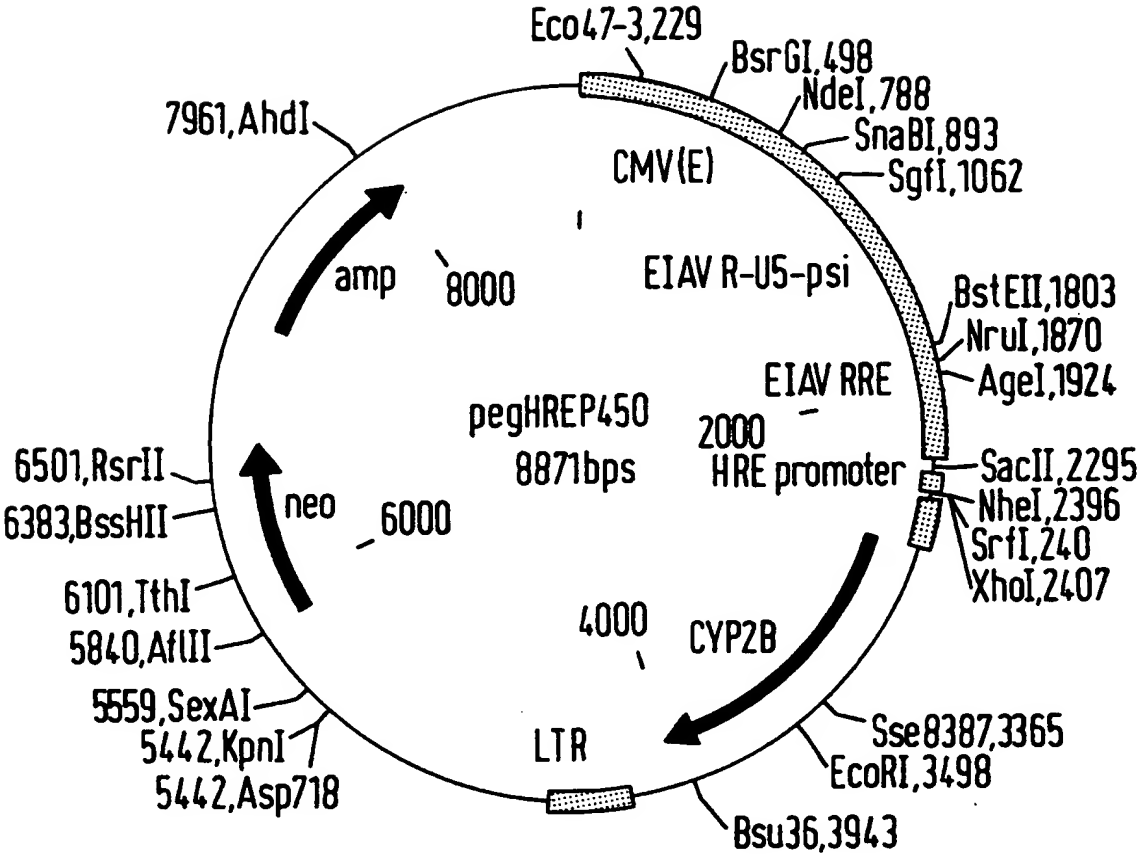

Eco47-3,229
BsrGI,498
NdeI,788
SnaBI,893
SgfI,1062
7961,AhdI
CMV(E)
amp
8000
EIAV R-U5-psi
BstEII,1803
NruI,1870
AgeI,1924
EIAV RRE
pegHREP450
8871bps
2000
HRE promoter
SacII,2295
NheI,2396
SrfI,240
XhoI,2407
6501,RsrII
6383,BssHII
neo
6000
6101,TthI
5840,AflII
4000
CYP2B
5559,SexAI
5442,KpnI
5442,Asp718
LTR
Sse8387,3365
EcoRI,3498
Bsu36,3943

FIG.16

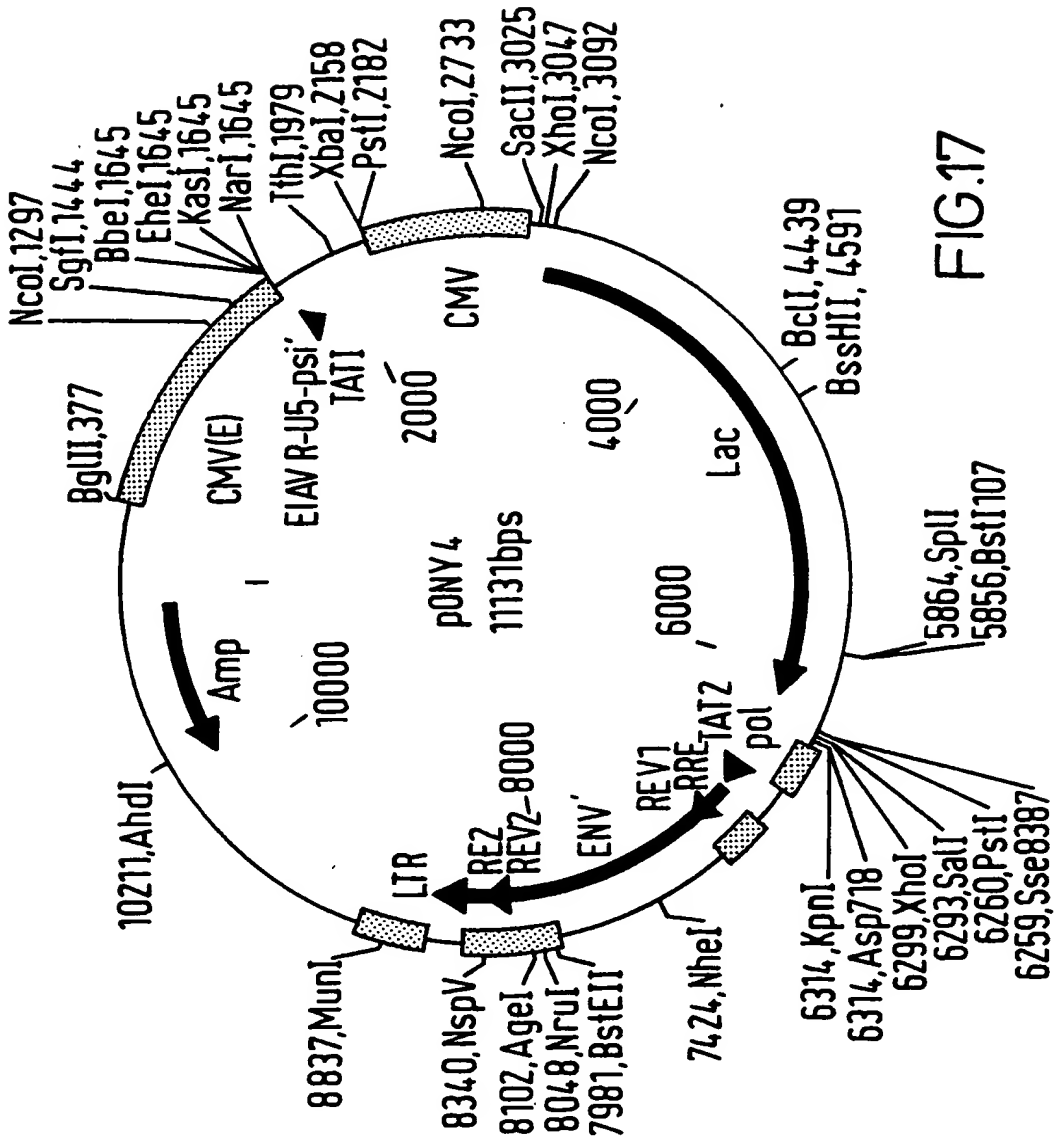

NcoI,1297
SgfI,1444
BbeI,1645
EheI,1645
KasI,1645
NarI,1645
TthI,1979
XbaI,2158
PstI,2182
NcoI,2733
SacII,3025
XhoI,3047
NcoI,3092
BclI,4439
BssHII,4591
5864,SplI
5856,BstI107
6314,KpnI
6314,Asp718
6299,XhoI
6293,SalI
6260,PstI
6259,Sse8387
7424,NheI
8102,AgeI
8048,NruI
7981,BstEII
8340,NspV
8837,MunI
10211,AhdI
BglII,377
CMV(E)
EIAV R-U5-psi'
TAT1
CMV
Lac
pol
TAT2
RRE
REV1
ENV'
REV2-8000
RE2
LTR
Amp
pONY4
11131bps
2000
4000
6000
10000

FIG.17

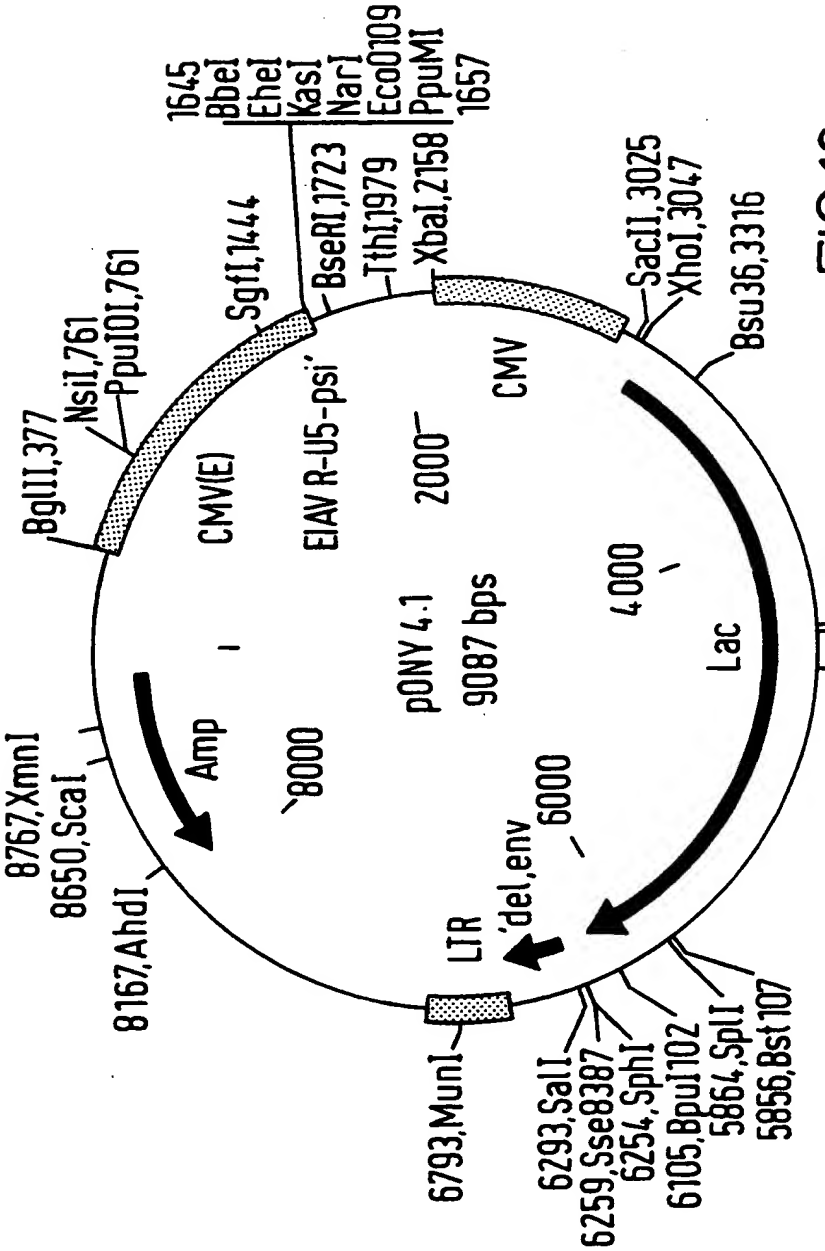

1645
BbeI
EheI
KasI
NarI
EcoO109
PpuMI
1657
BseRI,1723
TthI,1979
XbaI,2158
SacII,3025
XhoI,3047
Bsu36,3316
BsaBI,4420
BclI,4439
4591,BssHII
6793,MunI
6293,SalI
6259,Sse8387
6254,SphI
6105,BpuI102
5864,SplI
5856,Bst107
8767,XmnI
8650,ScaI
8167,AhdI
BglII,377
NsiI,761
PpuI01,761
Sg1I,1444
CMV(E)
EIAV R-U5-psi'
CMV
Amp
LTR
'del,env
Lac
pONY 4.1
9087 bps
2000
4000
6000
8000

FIG.18

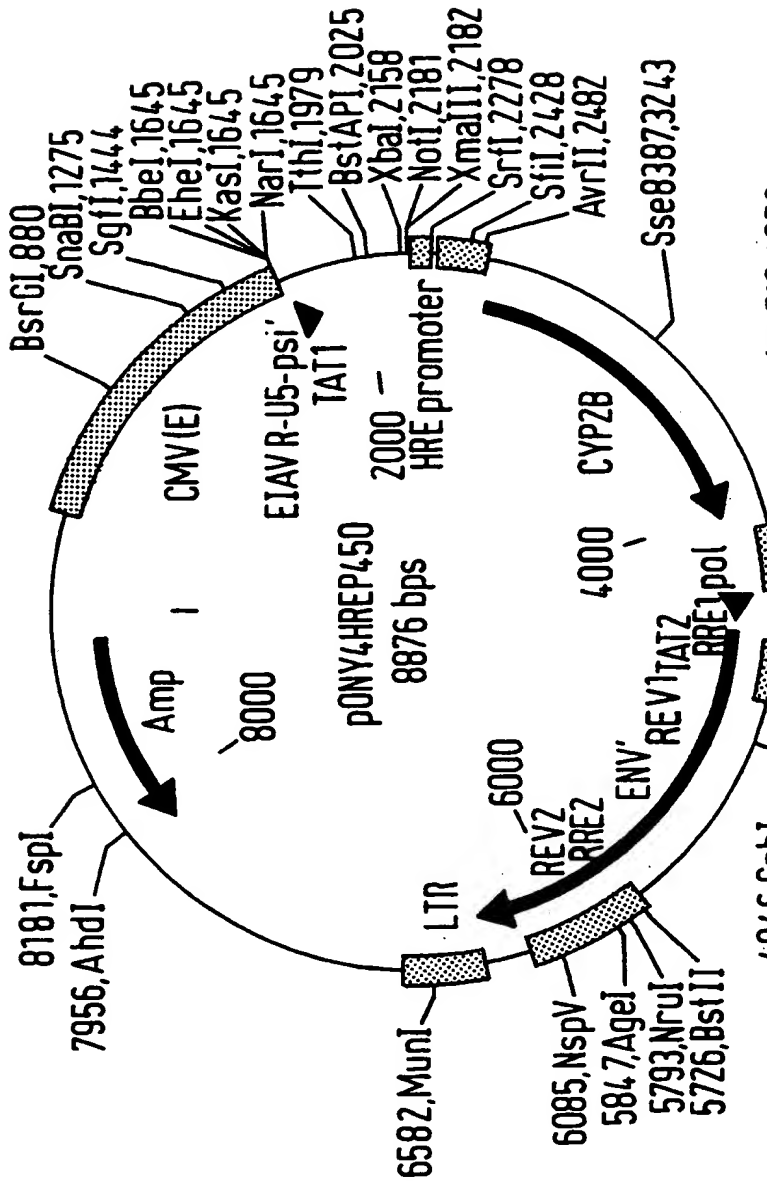

BsrGI,880
SnaBI,1275
SgfI,1444
BbeI,1645
EheI,1645
KasI,1645
NarI,1645
TthI,1979
BstAPI,2025
XbaI,2158
NotI,2181
XmaIII,2182
SrfI,2278
SfiI,2428
AvrII,2482
Sse8387,3243
Asp718,4059
KpnI,4059
4946,SphI
4760,BpuI 102
6085,NspV
5847,AgeI
5793,NruI
5726,BstII
6582,MunI
8181,FspI
7956,AhdI
CMV(E)
EIAV R-U5-psi'
TAT1
2000
HRE promoter
CYP2B
4000
RRE1
pol
TAT2
REV1
ENV'
6000
REV2
RRE2
LTR
Amp
8000
pONY4HREP450
8876 bps

FIG.19

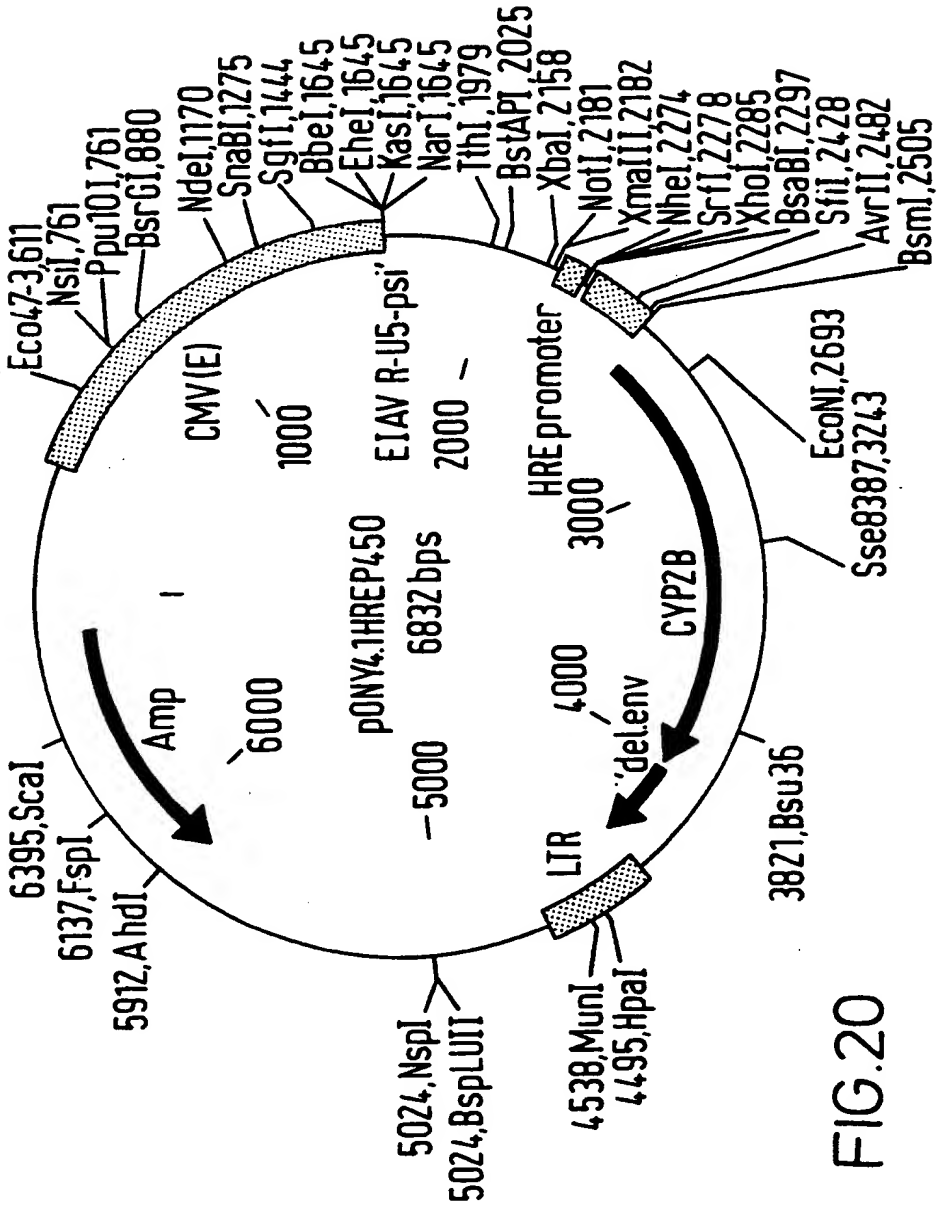

pONY4.1HREP450
6832 bps
CMV(E)
EIAV R-U5-psi
HRE promoter
Amp
'del.env
LTR
CYP2B
1000
2000
3000
4000
5000
6000
Eco47-3,611
NsiI,761
Ppu10I,761
BsrGI,880
NdeI,1170
SnaBI,1275
SgfI,1444
BbeI,1645
EheI,1645
KasI,1645
NarI,1645
TthI,1979
BstAPI,2025
XbaI,2158
NotI,2181
XmaIII,2182
NheI,2274
SrfI,2278
XhoI,2285
BsaBI,2297
SfiI,2428
AvrII,2482
BsmI,2505
EcoNI,2693
Sse8387,3243
3821,Bsu36
4538,MunI
4495,HpaI
5024,NspI
5024,BspLUII
5912,AhdI
6137,FspI
6395,ScaI

FIG.20

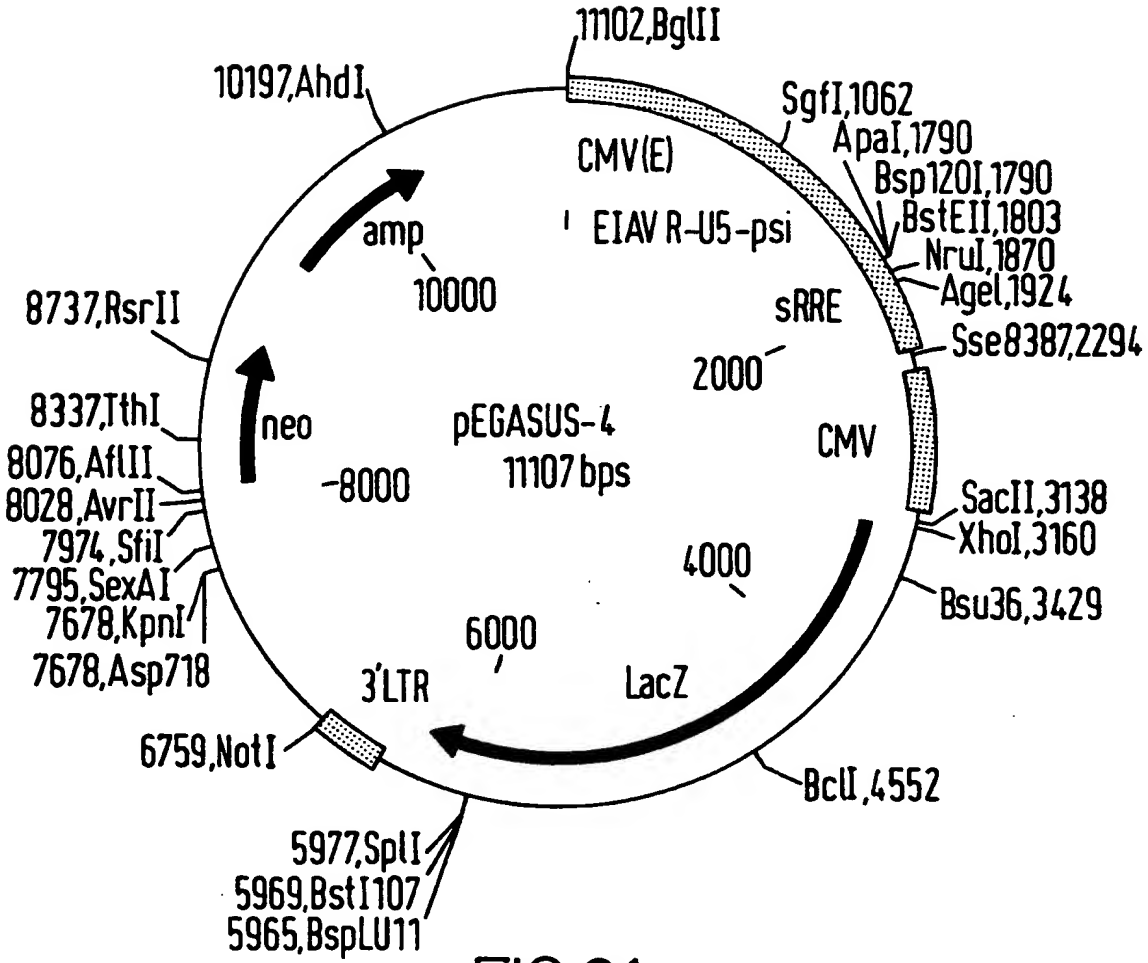

11102,BglII
10197,AhdI
SgfI,1062
ApaI,1790
Bsp120I,1790
BstEII,1803
NruI,1870
AgeI,1924
Sse8387,2294
CMV(E)
EIAV R-U5-psi
amp
10000
sRRE
2000
8737,RsrII
8337,TthI
8076,AflII
8028,AvrII
7974,SfiI
7795,SexAI
7678,KpnI
7678,Asp718
neo
pEGASUS-4
11107 bps
8000
CMV
SacII,3138
XhoI,3160
4000
Bsu36,3429
6000
3'LTR
LacZ
6759,NotI
BclI,4552
5977,SplI
5969,BstI107
5965,BspLU11

FIG.21

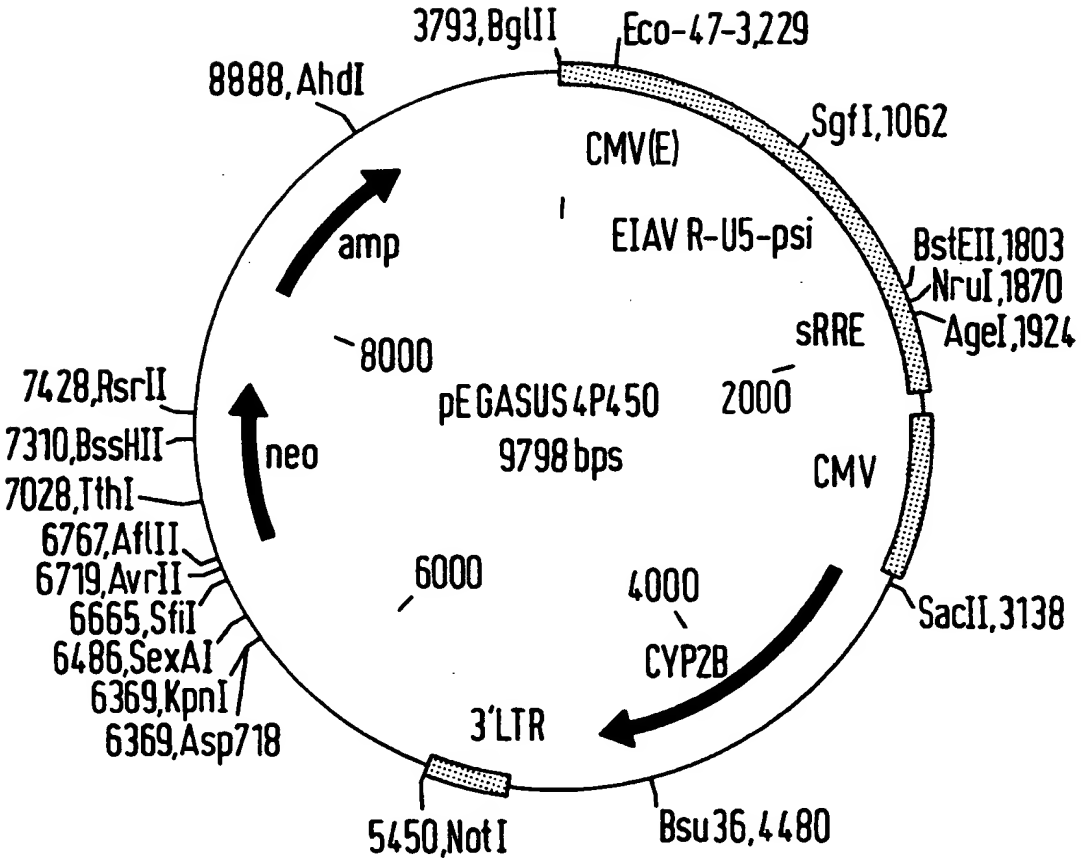

3793,BglII
Eco-47-3,229
8888,AhdI
SgfI,1062
CMV(E)
EIAV R-U5-psi
amp
BstEII,1803
NruI,1870
AgeI,1924
sRRE
8000
2000
7428,RsrII
pEGASUS4P450
9798 bps
7310,BssHII
neo
CMV
7028,TthI
6767,AflII
6719,AvrII
6000
4000
SacII,3138
6665,SfiI
CYP2B
6486,SexAI
6369,KpnI
3'LTR
6369,Asp718
5450,NotI
Bsu36,4480

FIG.22

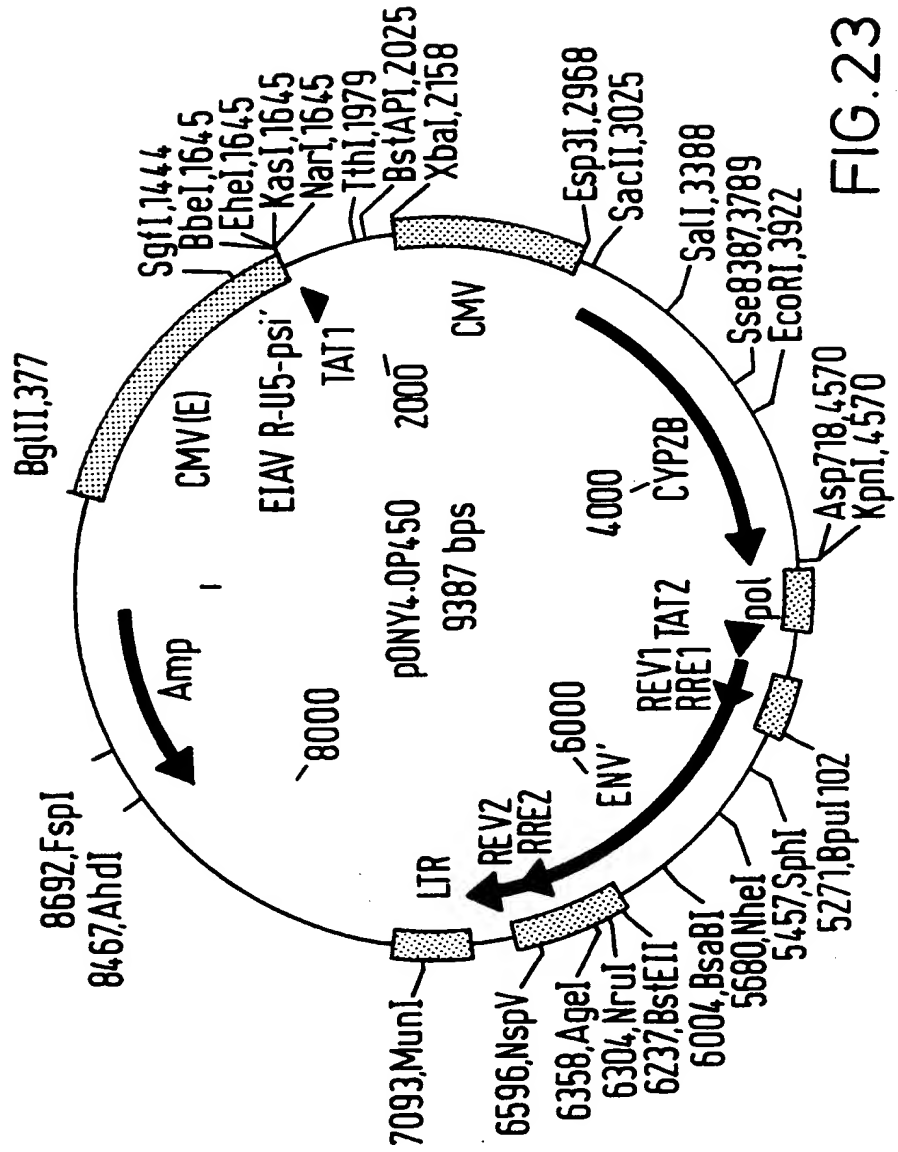

pONY4.0P450
9387 bps
2000
4000
6000
8000
CMV
CMV(E)
EIAV R-U5-psi'
TAT1
TAT2
REV1
RRE1
REV2
RRE2
ENV'
pol
LTR
Amp
Sgf1,1444
BbeI,1645
EheI,1645
KasI,1645
NarI,1645
TthI,1979
BstAPI,2025
XbaI,2158
Esp3I,2968
SacII,3025
SalI,3388
Sse8387,3789
EcoRI,3922
Asp718,4570
KpnI,4570
5271,BpuI102
5457,SphI
5680,NheI
6004,BsaBI
6237,BstEII
6304,NruI
6358,AgeI
6596,NspV
7093,MunI
8467,AhdI
8692,FspI
BglII,377

FIG.23

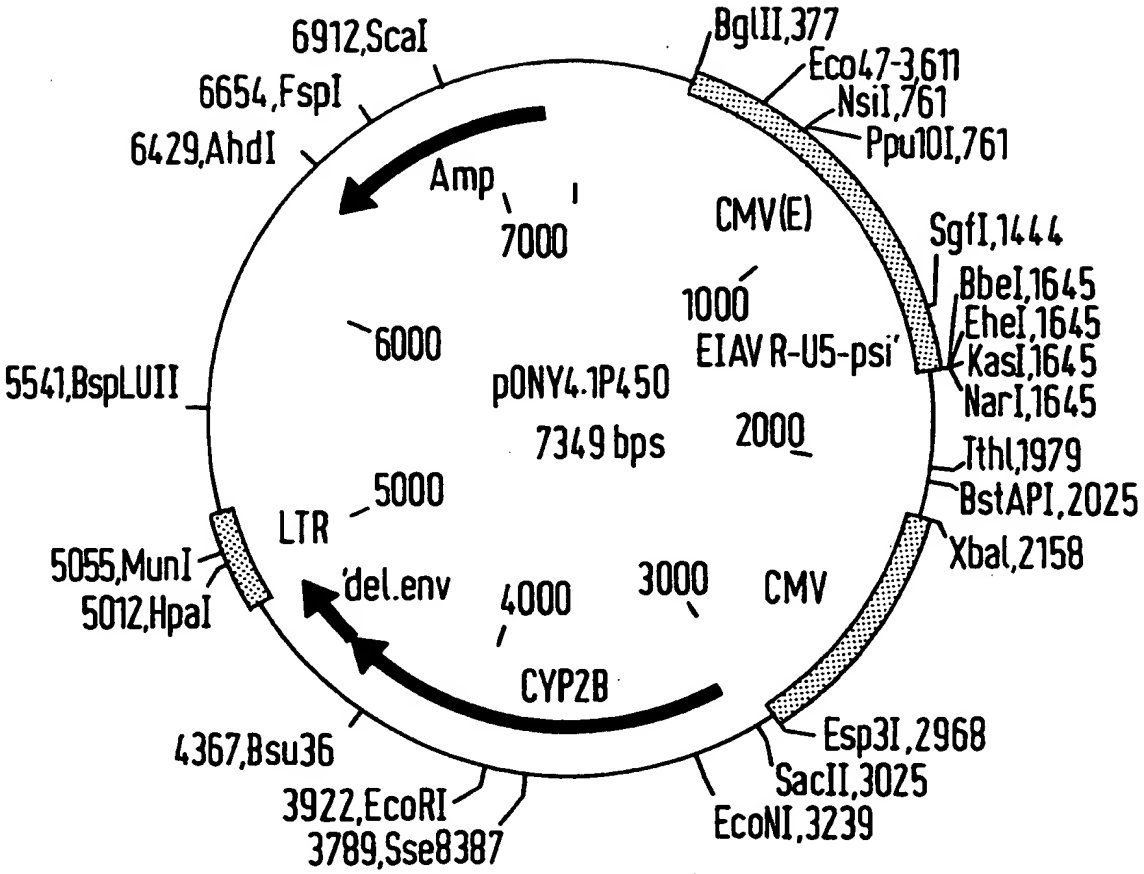

6912,ScaI
6654,FspI
6429,AhdI
Amp
7000
BglII,377
Eco47-3,611
NsiI,761
Ppu10I,761
CMV(E)
1000
SgfI,1444
BbeI,1645
EheI,1645
KasI,1645
NarI,1645
EIAV R-U5-psi'
6000
5541,BspLUII
pONY4.1P450
7349 bps
2000
ItthI,1979
BstAPI,2025
5000
LTR
XbaI,2158
5055,MunI
5012,HpaI
'del.env
4000
3000
CMV
CYP2B
Esp3I,2968
SacII,3025
4367,Bsu36
3922,EcoRI
3789,Sse8387
EcoNI,3239

FIG. 24